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Volume 32 Number 2

ENDOCRINOLOGY

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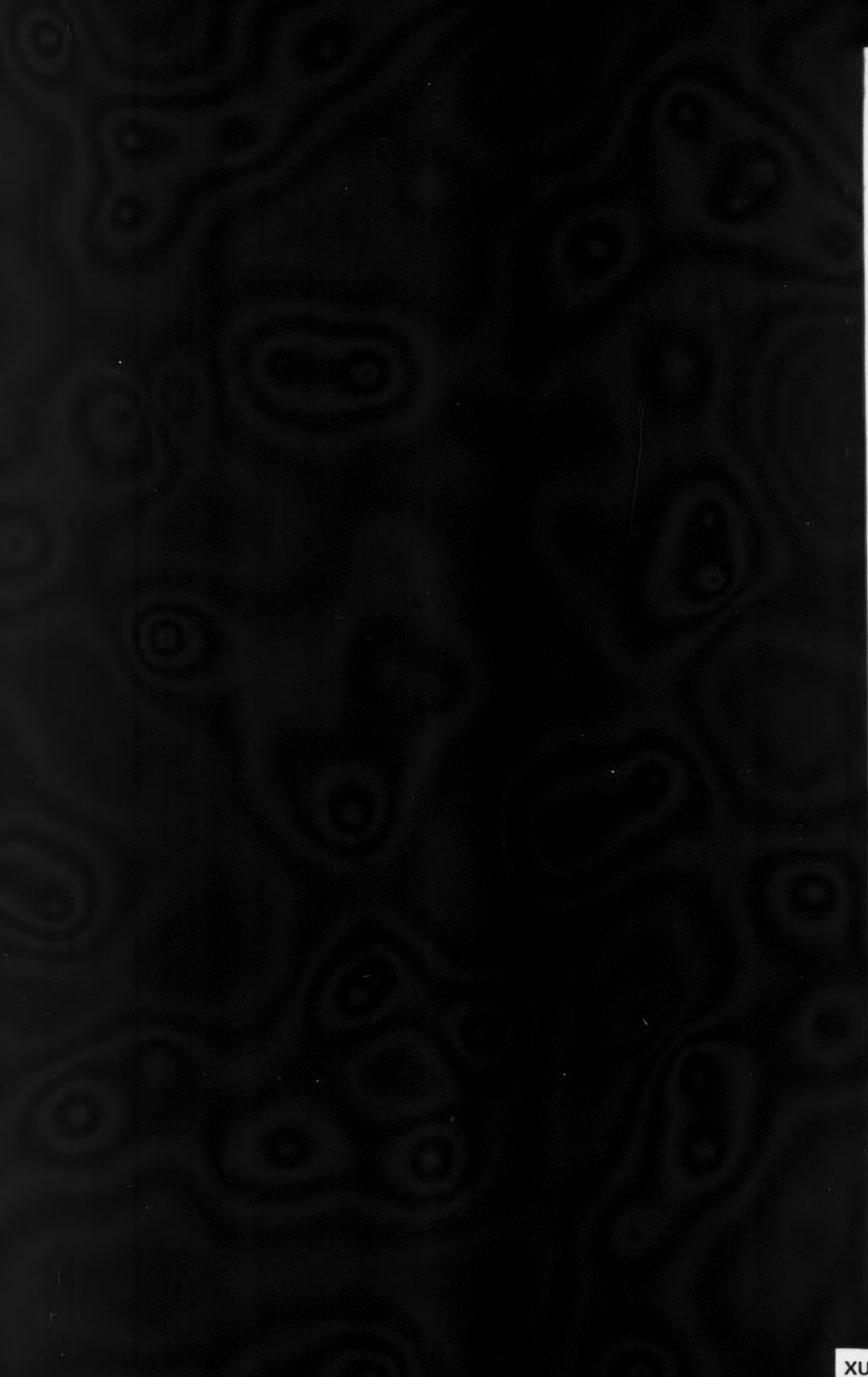

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NATIONAL RESEARCH COUNCIL GRANTS FOR RESEARCH IN ENDOCRINOLOGY

IT IS ANNOUNCED that requests to the National Research Council Committee for Research in Endocrinology for aid during the fiscal period from July 1, 1943 to June 30, 1944 will be received until February 28, 1943. Application blanks may be obtained by addressing the Division of Medical Sciences, National Research Council, 2101 Constitution Avenue, Washington, D.C. In addition to a statement of the problem and research plan or program, the Committee desires information regarding the proposed method of attack, the institutional support of the investigation and the uses to be made of the sum requested. No part of any grant may be used by the recipient institution for administrative expenses. Applications for aid of endocrine research on problems of sex in the narrower sense cannot be given favorable consideration, but the Committee will consider support of studies on the effects of sex hormones on non-sexual functions—e.g., on metabolism.

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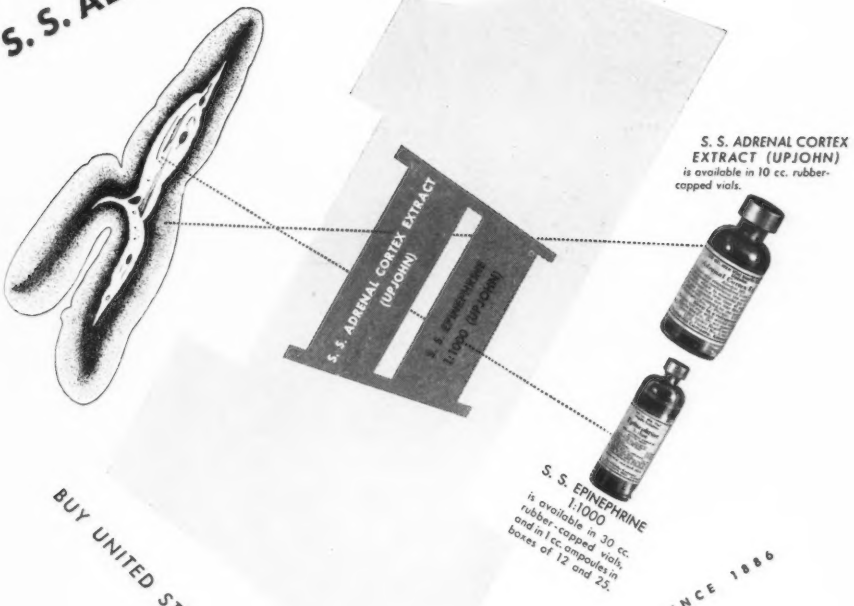

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ENDOCRINOLOGY

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ENVIRONMENTAL MODIFICATION OF CERTAIN ENDOCRINE PHENOMENA[1]

EDWARD W. DEMPSEY AND HAROLD F. SEARLES

From the Department of Anatomy, Harvard Medical School

BOSTON, MASSACHUSETTS

IT HAS long been known that certain endocrine readjustments may be caused by exteroceptive stimuli. Examples may be found in many of the endocrine glands. The yearly growth and regression of the sexual organs in animals in which reproduction is seasonal are closely correlated with the external temperature and light conditions (1, 2). The act of mating, as well as mechanical stimulation of erogenous zones, is necessary for the induction of ovulation in the rabbit and pseudopregnancy in the rat. Exposure to cold causes an increase in the basal metabolic rate, in the thyroid cell height, and in the weight of the adrenal cortex. Conversely, maintenance in a hot environment has been observed to lower the metabolic rate (3). These observations all support the generalization that important changes in endocrine activity can result from changes in the external world.

Such observations have prompted a re-examination of the endocrine responses to several environmental stimuli. It was desired to examine more rigorously in a single species certain observations originally made upon different species and under different experimental conditions. This procedure, it was hoped, might yield data of sufficient quantitative accuracy to permit certain generalizations not otherwise possible. Furthermore, these data could be used as a point of departure for studies on the neurological pathways utilized by the environmental stimuli. In this last connection it was specifically of interest to determine the effect of transection of the pituitary stalk upon the various phenomena, since it has recently been reported that this operation prevents the appearance of at least two endocrine responses attributed to exteroceptive stimulation. In the present paper the reactions of the thyroid, adrenals, and ovaries of rats exposed to light, heat, cold, and sterile mating, together with the effects of pituitary stalk section on the various phenomena, will be described.

MATERIAL AND METHODS

Albino rats, secured through a local animal breeder, were used. In 60 female animals, weighing 120 to 150 gm., the pituitary stalk was sectioned by the parapharyngeal approach (4). After a determination of the regularity of reproductive cycles and gain in weight, the animals were used in the various experimental procedures described in the following sections.

Received for publication September 11, 1942.

[1] This investigation was supported by a grant from the Committee for Research in Problems of Sex, National Research Council.

Pseudopregnancy was induced by mating females with vasectomized males. This procedure was chosen since it is more nearly the physiologic stimulus than glass rod or electrical stimulation of the cervix. Exposure to cold was accomplished by placing animals in their regular cages in a well-ventilated refrigerator which was maintained at a temperature of 35 to 40°F. A heated environment was provided by a large gas-heated and water-jacketed paraffin oven into which the regulation cages were placed. The temperature was maintained by means of a thermostat at 95 ± 3°F. This temperature was selected on the basis of preliminary experiments which showed that the body temperature rose and death resulted when higher temperatures were utilized. Stimulation by light was achieved by maintaining rats in an enclosure illuminated constantly by tungsten-filament, frosted-glass bulbs. The light intensity, measured at various points inside the cages with a General Electric photometer, was between 60 and 90 foot candles. Maintenance of the light enclosure at room temperature, despite the heat produced by the lamps, was accomplished by placing the light enclosure in front of a ventilating duct so that the room air of the ventilating system passed through it.

At the end of each experimental period the rats were killed by decapitation. The thyroids and ovaries were fixed in Bouin's fluid and sectioned after embedding in paraffin. The adrenals were dissected clean and weighed to the nearest milligram. The ratio of adrenal weight/body-surface was then determined. The thyroid cell-height index (5) was computed. These indices, in the experimental animals, were expressed as percentages of the values observed in control animals which had been maintained under normal room conditions. The degree of ovarian follicular and luteal development was noted.

In the case of the animals in which the stalk had been transected, the skull was opened and the entire head fixed in 10 per cent formalin. Partial decalcification was produced by treatment with 5 per cent tri-chloracetic acid. A block containing the pituitary region was prepared which was then completely decalcified. The block was serially sectioned in the sagittal plane through the stalk region. This was accomplished by frozen sections in some instances, and by celloidin methods in others. Finally, alternate sections were stained with hematoxylin and eosin and Bodian's protargol method. The sections were examined for completeness of transection, and only the animals in which the operation was successful are included in this report.

RESULTS

Reproductive cycle after pituitary stalk transection. Of the 60 rats operated and observed subsequently for periods of 1 to 6 months, only 2 remained permanently in diestrus. The cycles of the remainder were arbitrarily classified as regular or irregular after inspection of the contour charts of their vaginal cycles. Twenty-eights rats showed regular cycles throughout the observation period, while 30 were more or less irregular. These irregularities were classified in three groups. *a)* Eleven animals of the 30 showed long diestrous intervals at first, but their cycles became more normal within one month after the operation. *b)* Thirteen animals had diestrous intervals of 10 days or longer, which occurred spontaneously and repeatedly. *c)* The remaining 6 animals can be characterized only by the statement that the cyclic vaginal cornification was completely unpredictable. This group of irregular animals was larger than that previously described (6), although in the present series a smaller percentage of animals remained permanently in diestrus.

Induction of pseudopregnancy after pituitary stalk section. Ten of the rats in which the reproductive cycles were regular after stalk section were caged with vasectomized males. After 1 to 4 normal cycles, all of the 16 rats showed the prolonged diestrous period characteristic of pseudopregnancy (fig. 1). In order to test whether this dies-

trous interval represented pseudopregnancy, the females were allowed to remain caged with the males until the end of the next estrous period. On the fourth day following the appearance of cornification, under ether anesthesia the uterus was traumatized by the introduction of a loop of silk thread. On the fourth day after this treatment the uterus was again exposed and the presence or absence of deciduomata was noted. In 9 of the 10 animals large decidual enlargements were observed.

The luteal function in this group of animals was also tested by observing the reaction to large doses of estrogen (7). During a pseudopregnant interval an injection of

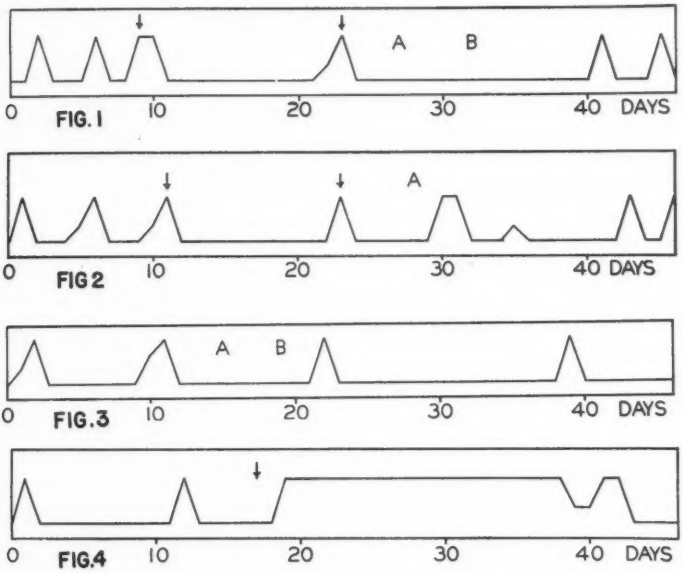


Fig. 1. Estrous cycles and induction of pseudopregnancy with decidual reaction in a female rat with sectioned pituitary stalk. Sterile copulation, indicated by arrows, was followed by prolonged diestrous intervals. At *A*, uterine trauma produced a decidual reaction observed by laparotomy at *B*. This and following records are typical of similar experiments on groups of animals, the number of which is given in the text.

Fig. 2. Effect of large injection of estrogen during pseudopregnancy. Sterile copulation, indicated by arrows, was followed by prolonged diestrus. At *A* an injection of 2500 I.U. of estradiol benzoate was followed by a short period of vaginal cornification, after which the animal again reverted to diestrus.

Fig. 3. Failure of production of decidual reaction during spontaneously long cycles in rat with sectioned pituitary stalk. No decidual reaction was revealed by laparotomy at *B* after uterine trauma at *A*.

Fig. 4. Prolonged effect of large injection of estrogen during spontaneously long cycles in rats with sectioned pituitary stalk. At arrow, an injection of 2500 I.U. of estradiol benzoate was followed by prolonged vaginal cornification. Compare with figure 2.

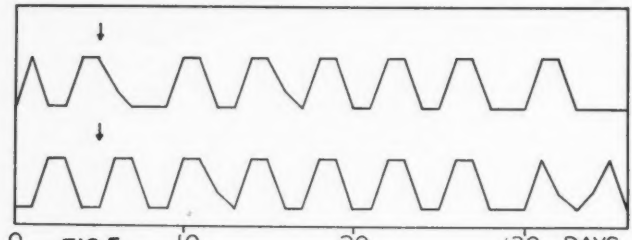
2500 I.U. of estradiol benzoate[2] produced either no effect or a period of cornification of 1 to 4 days in duration (fig. 2).

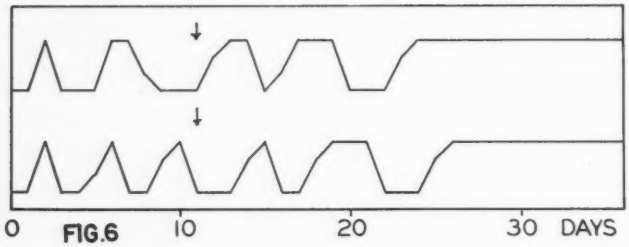
Similar tests for luteal function were made in 10 animals in which spontaneously prolonged cycles were observed after stalk section (fig. 3). A decidual reaction was observed in only 1 of the 10 when the uteri were threaded 4 days after the last estrous period. Likewise, the reaction of these animals to estrogen treatment was very different from those in which the long cycles had been induced by sterile mating. Six of

[2] The estradiol benzoate (Progynon-B) was supplied by the Schering Corp., Bloomfield, N. J.

the 10 animals showed prolonged periods of cornification as a result of the estrogen treatment (fig. 4).

The data cited in the preceding paragraph indicate that the spontaneously long cycles in animals in which the pituitary stalk was cut, although superficially similar to pseudopregnant cycles, are characterized by quite different endocrinological reactions. It remained to be demonstrated, however, whether true pseudopregnancy could be induced in such animals. Consequently, 5 of the 10 rats were mated with vasectomized males and tested by estrogen injection during the cycle following the sterile mating. The periods of cornification induced were short (1 to 3 days) in all 5 animals, indicating that pseudopregnancy had occurred.





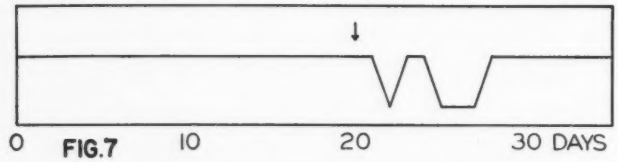


Fig. 5. LACK OF EFFECT OF CONFINEMENT IN HOT ENVIRONMENT (begun at arrows) on the reproductive cycle. *Upper record*, normal female rat; *lower record*, female rat with severed pituitary stalk.

Fig. 6. EFFECT OF EXPOSURE TO CONSTANT LIGHT (begun at arrows) on the reproductive cycle. *Upper record*, normal female rat; *lower record*, female rat with sectioned pituitary stalk.

Fig. 7. EFFECT OF A SINGLE INJECTION (at arrow) of pregnancy urine concentrate upon constant cornification induced by light.

Endocrine reactions during exposure to heat. Six normal and 6 female rats in which the pituitary stalk was cut were placed in a hot box at a temperature of 95°F. and maintained there for a period of 32 days. During this time the reproductive cycles were relatively unaffected, the only alteration being a slight prolongation of the diestrous intervals in 2 of the rats with the severed stalk (fig. 5). The ovaries in all animals appeared normal and contained recent corpora lutea. The adrenals appeared normal, and the ratio of adrenal weight to body surface was within normal limits.

The thyroid, however, showed histologic evidence of colloid storage and the follicle cells were flattened. The cell-height index was 73 and 70 per cent of the control value for the normal and operated animals, respectively.

Endocrine reactions during exposure to cold. The effects of exposure to cold have been described previously (4, 6), and need not be repeated in detail here. Three normal and 7 of the rats with a severed pituitary stalk in which pseudopregnancy had been induced previously were confined in a cold-room at 35°F. for 6 days. The adrenal weight/body surface ratios were 119 and 108 per cent for the normal and operated groups, respectively. The thyroid cell-height index was, in the same order, 141 and 102 per cent.

Endocrine effects during exposure to light. Six normal and 6 female rats with severed pituitary stalks were placed in a light enclosure where they were maintained for one month. During this time the periods of vaginal cornification became longer and longer until finally the cyclical vaginal changes ceased in continuous cornification (fig. 6). Examination of the ovaries revealed in all cases large and medium-sized follicles, but no corpora lutea (cf. 8, 9). In the operated group the adrenal weight/body surface ratio was somewhat depressed (85 per cent) while the ratio for the normal group was 99 per cent. The thyroids were histologically similar to those from animals exposed to heat. Cell-height indices of 68 per cent were recorded for both the normal and the operated groups.

Since the constant vaginal cornification induced by exposure to light was associated with a failure of ovulation, experiments were undertaken in a effort to induce ovulation. In a preliminary experiment, 6 animals exhibiting constant cornification were given a single subcutaneous injection of 50 R.U. of pregnancy urine concentrate.[3] In 4 of the 6 females a transient diestrus lasting 1 to 3 days was produced, following which the animals had one or more cycles before the vaginal smear returned to the constantly cornified state (fig. 7). No effect was observed in the remaining 2 animals.

In order to determine whether or not the cyclic interruption of the constant cornification produced by the pregnancy urine was associated with ovulation, the same 6 rats were again given an injection of pregnancy urine. As before, 5 of the 6 injected animals showed diestrous smears on the day after the injection. Two of these animals were killed and fresh corpora lutea were observed in the sectioned ovaries. The 3 remaining rats were killed 5 and 10 days after the injection, and examination of the ovaries showed 2 or 3 sets of corpora lutea. These experiments permit the conclusions, therefore, that the large follicles which are continuously present during prolonged exposure to constant light can be caused to ovulate by the introduction of luteinizing substances, and that the corpora lutea so formed are of the cyclic type in that pseudopregnancy is not associated with their presence.

The possibility also existed that the stimulus of mating, which ordinarily leads to pseudopregnancy in the rat, might cause ovulation of the large continuously present follicles of animals exposed to light. Consequently, 6 normal females, in which constant vaginal cornification had been induced, were caged with vasectomized males. In 5 of the 6, the vaginal smears became diestrous a day later and remained so for periods of 12 to 18 days. At the end of this interval, periodic cornification occurred for 2 or 3 cycles after which constant vaginal cornification again ensued (fig. 8).

In order to test more rigorously whether or not ovulation and prolonged luteal function were associated with the prolonged diestrous interval which sterile mating evoked in animals exposed to light, the above experiment was extended by

[3] The pregnancy urine gonadotropin (antuitrin-S) was supplied by Parke, Davis & Co., Detroit, Mich.

observation of the ovaries and by threading the uterus in an additional group of 5 animals. All 5 animals displayed diestrous smears after mating, and, on laparotomy 4 days after mating, corpora lutea were observed in the ovaries. The uteri were threaded at this operation and 8 days after mating the animals were killed. Deciduomata were observed in the threaded uteri of all 5 animals. It can be concluded, therefore, that sterile mating is a sufficient stimulus to evoke ovulation and pseudopregnancy in rats exposed to constant light.

The dependence of ovulation upon the stimulus of copulation in rats exposed to light was of especial interest because of its similarity to the control of ovulation in species which do not spontaneously ovulate, such as the cat and rabbit. Furthermore,

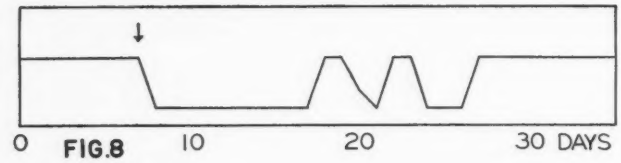


FIG.8

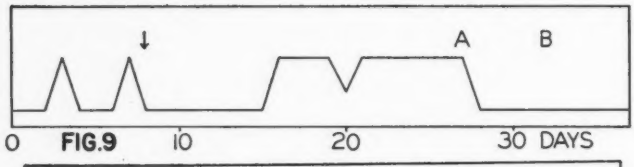


FIG.9

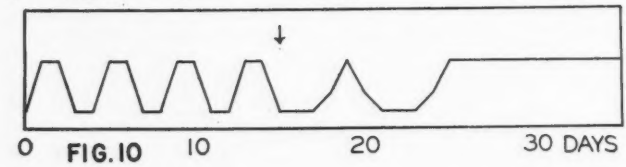


FIG.10

Fig. 8. Effect of sterile mating (at arrow) upon constant cornification induced by light.

Fig. 9. Induction of ovulation by sterile mating in female rat with sectioned pituitary stalk (A) during a period of constant cornification induced by exposure to light (begun at arrow). Laparotomy at *B* revealed fresh corpora lutea.

Fig. 10. Effect of light and cold (begun at arrow) upon the reproductive cycle of the rat.

since Brooks (10) found that the ovulatory mechanism in the rabbit failed to function after pituitary stalk section, it was of interest to inquire whether the experimentally induced mechanism in the rat also depends upon the intact pituitary stalk. For these reasons, 4 female rats with severed pituitary stalks were caged in the light enclosure until the cyclic reproductive activity changed into continuous vaginal cornification. The animals were then caged with vasectomized male rats. Prolonged diestrous intervals were produced in 3 of the 4 females (fig. 9). Moreover, laparotomy revealed the presence of fresh corpora lutea, and uterine threading induced decidual reactions during the prolonged diestrus. It is, therefore, apparent that the pituitary stalk is not essential for the production of copulation-induced ovulation and pseudopregnancy in female rats.

Endocrine effects during exposure to light and cold. Six normal female rats were placed in a constantly lighted refrigerator and maintained there for one month. Al-

terations appeared in the reproductive cycles which were entirely comparable to those produced by exposure to light alone (fig. 10). Similarly, large follicles and no corpora lutea were observed on examination of ovarian sections. The adrenals appeared grossly enlarged and pigmented, and the adrenal weight/body surface ratio was 120 per cent. The thyroid glands also showed an increase in the cell-height index (140 per cent) besides the histologic picture ordinarily associated with increased activity.

DISCUSSION

The experiments described above, together with the older reports referred to in the introduction, abundantly demonstrate that endocrine activity may be modified by exteroceptive stimuli. Moreover, since the exposure to light, heat and cold, to which the rats were subjected in the present experiments, is comparable to the natural environmental range of these factors for the rat, the inference is clear that the concomitant changes in endocrine activity represent normal fluctuations of the 'endocrine balance.' Endocrine balance is not, therefore, a static, specific endocrine interrelationship, but rather it is a dynamic state capable of varying according to the particular constellation of stimuli operative at any instant.

The endocrine changes observed in the present investigation may be stated in terms of fluctuations of specific anterior pituitary principles. An increase or decrease in thyroid cell height presumably reflects a corresponding change in concentration of the circulating thyrotropic hormone. Similarly, changes in weight of the adrenal gland represent corresponding changes in adrenotropic activity. In regard to the ovary, constant follicular development coupled with continuous estrogen production may be taken as indicative of combined follicle stimulating and luteinizing hormone action, since it has been shown (11) that changes in the FSH/LH ratio cause variations in estrogen output. The observations on ovulation, however, permit more definite statements as to the rate of release of the luteinizing hormone. A relatively sudden increase in concentration of this latter hormone causes ovulation in a wide variety of species, and in various experimental situations characterized by the presence of large follicles. Thus, follicular rupture is induced by injections of luteinizing substances into normal estrous rabbits (12) and into rats during constant estrus, whether the constant estrus is spontaneous (13) or induced either by prepuberal testicular grafts (14) or by long-continued exposure to light. A like result also is obtained in guinea pigs during pregnancy or after long-continued progesterone treatment (15). Consequently, it seems possible to conclude that a change in thyroid cell height or in adrenal weight reflects a corresponding change in thyrotropic or adrenotropic hormone production, and that constant estrus reveals a constant low level of luteinizing hormone activity.

By application of the considerations mentioned above, it becomes possible to define the effects of light, heat and cold in terms of the anterior pituitary fractions. Exposure to heat entails a reduction in thyroid cell height and therefore represents a decrease in thyrotropic hormone production. Cold increases the adrenotropic and thyrotropic effects. Light depresses the thyrotropic and luteinizing hormone concentrations. Light and cold together increase the thyrotropic and adrenotropic hormones, while causing a decrease in effect of the luteinizing hormone. Lastly, in rats in which the pituitary stalk is cut, cold increases the adrenotropic concentration.

Taken together, the results cited above show that with respect to the adrenotropic, thyrotropic and luteinizing fractions, any one of these may remain at a fixed level or may vary quite independently of the degree of activity expressed by the other two (table 1). The endocrine balance of the individual animal is not only a dynamic

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equilibrium, therefore, but also an equilibrium in which there is a considerable freedom of movement of the constituent elements.

The principle that the adrenotropic, thyrotropic and luteinizing hormones each can vary in rate of production independently of the other two throws light on the question of the form of secretion of the anterior pituitary hormones. Since only two secretory cell types, the basophils and the acidophils, are ordinarily recognized on cytologic grounds, the suspicion has been prevalent that the multiplicity of pituitary effects might be a methodological artifact. According to this argument, the pituitary might secrete only two substances, each of which might be capable of exerting different effects depending upon the end organ it chanced to reach. This point of view has been supported by the clinical observation that pituitary diseases tend to produce multiglandular overactivities of two main types, and that the particular constellation of symptoms in each type is correlated with disorders of the acidophils or basophils. The principal refutation of this argument has been the demonstration that a separation of the pituitary effects could be made on a basis of chemical fractionation. Nevertheless, such chemical data are not entirely conclusive because, conceivably, a

TABLE 1. EFFECT OF VARIOUS EXPERIMENTAL TREATMENTS UPON RATES OF PRODUCTION OF 3 PITUITARY FRACTIONS. INCREASED RATES ARE INDICATED BY PLUS SIGNS, DECREASED RATES BY MINUS SIGNS, NO DETECTABLE CHANGE, BY ZEROS.

Experimental Treatment	Adrenotropic Effect	Thyreotropic Effect	Luteinizing Hormone Effect
Heat	0	−	0
Light	0	−	−
Light and cold	+	+	−
Cold	+	+	0
Cold (stalk cut)	+	0	0

large molecule with several prosthetic groups could be disintegrated and separated into several active fractions as a result of the chemical treatment. This objection does not apply to the experiments here reported, since the separability of the effects was determined on a basis of simultaneously estimated degrees of activity of the several responses, as a result of physiologic stimulation and secretion. Consequently, it can be stated that the pituitary secretes at least 3 qualitatively different substances at independent rates.

The present experiments on the effects of sterile mating were undertaken in an effort to clarify the factors involved in the production of ovulation and pseudopregnancy. There is evidence, but not general agreement, that both phenomena can occur after the transection of pituitary stalk in such spontaneously ovulating forms as the guinea pig (16) (but cf. Dey, 17) the monkey (18), and the human being (19). On the other hand, stalk transection prevents coitus-induced ovulation in the rabbit which does not spontaneously ovulate (10). Pseudopregnancy is apparently unaffected by stalk section in this latter species, however, since Brooks (10) found that the induction of ovulation by injections of luteinizing substances was followed by progestational changes which were normal in character and duration.

The reproductive cycle of the rat is peculiar in that ovulation is spontaneous, but the stimulus of mating normally is required for the initiation of luteal function. In this species, transection of the pituitary stalk does not prevent ovulation, pregnancy, parturition or lactation (6, 20). Normal pregnancy, however, does not necessarily indicate that the luteal activity necessary for implantation had been induced

by mating. Richter (21) reported that the reproductive cycles were prolonged in rats in which the pituitary stalk was cut. The prolonged cycles usually were a multiple of the normal cycle, and he therefore suggested that pseudopregnant cycles were the rule after stalk section. If this were true, the production of pregnancy in rats with severed pituitary stalks could be explained without recourse to a stimulus for pseudopregnancy, since luteal function with a consequent preparation of the uterus for implantation would automatically follow each ovulation.

Considerations derived from the data presented in the first two series of experiments show that the above explanation does not apply. More than half of the animals with severed stalks had normal rather than prolonged cycles. Pseudopregnant cycles are, therefore, not an invariable consequence of stalk section. Moreover, the prolonged cycles which occurred in some of the operated animals were not associated with the phenomena characteristic of pseudopregnancy. Decidual reactions could not be induced by uterine traumatization, and injections of estrogens were followed by prolonged vaginal cornification. The prolonged cycles are not, therefore, a result of prolonged luteal function. It is more probable that they are a consequence of pituitary deficiencies caused by incidental operative damage.

Since the above considerations show that pseudopregnant cycles do not occur uniformly in rats in which the pituitary stalk is cut, it follows that the luteal function, as revealed by pregnancy, decidual reactions and inhibition of estrogen effects, must have resulted from the coital stimuli. Furthermore, since such stimuli produce their effects equally well in normal and operated animals, it can be concluded that an intact pituitary stalk is not essential for the reception of the stimuli by the pituitary.

The ovarian reactions to continuing exposure to light consist of a failure of cyclical ovulation and the constant maintenance of follicles of ovulatory size. Rupture of these follicles results from mating or the injection of luteinizing substances. In effect, then, a non-spontaneously ovulating cycle quite similar to that of the normal rabbit has been produced. Nevertheless, despite the similarities, ovulation follows mating in the rat with severed pituitary stalk which has been exposed to light, whereas after section of the stalk in the rabbit ovulation is blocked.

The suggestion has been made that the pituitary stalk subserves emergency functions (22). Nevertheless, a variety of endocrine activities which are induced by environmental stimuli, but which do not require the pituitary stalk, are described in the present report. These effects include the decrease in thyroid-cell height during exposure to heat, the ovarian and vaginal changes induced by constant exposure to light, the production of coitus-induced ovulation in animals exposed to light, and the production of pseudopregnancy. To this list may be added the adrenal enlargement on exposure to cold (4). On the other hand, coitus-induced ovulation in the rabbit and the increase in cell height of the rat thyroid during exposure to cold are prevented by stalk section. Nevertheless, the variety of the phenomena for which the pituitary stalk is not essential suggests that the participation of this structure in neuro-endocrine hypophysial effects may prove the exception rather than the rule. It follows, since activation of the anterior pituitary by fibers running in the pituitary stalk cannot be invoked as a general explanation, that the problem of the mechanism of neuro-endocrine control must still be regarded as an open question.

SUMMARY

Study of the vaginal cycles of a group of female rats after transection of the pituitary stalk has shown that unaltered, normal cycles are demonstrable in approximately half of the animals. Pseudopregnancy, as indicated by prolonged diestrus, produc-

tion of deciduomata, and short periods of vaginal cornification after injections of estrogens, was produced in these rats with sectioned pituitary stalk.

Approximately one-fifth of the operated animals displayed prolonged diestrous intervals. These prolonged cycles cannot be characterized as pseudopregnant cycles, however, since in this group the above-mentioned tests for luteal function were negative.

Maintenance of female rats in variously altered environments has shown that constant light causes constant follicular development without ovulation. Ovulation and pseudopregnancy were induced, however, by sterile mating. High environmental temperatures caused a depression of the thyroid-cell height. Exposure to cold resulted in increased thyroid-cell height and enlarged adrenals. Simultaneous exposure to cold and light produced constant estrus, as well as adrenal and thyroid stimulation. All of these reactions, with the exception of the thyroid stimulation by cold, were produced in animals after pituitary stalk section.

These results show that environmental stimuli may be transmitted to the anterior pituitary by routes other than the pituitary stalk. Furthermore, they demonstrate that various endocrine components react in unitary fashions when the endocrine balance is altered.

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EFFECT OF ADRENAL CORTICAL EXTRACT AND CERTAIN STEROID HORMONES ON THE RESPONSE OF ADRENALECTOMIZED RATS TO INTRAPERITONEAL INJECTIONS OF AN ISOTONIC GLUCOSE SOLUTION

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THE INTRAPERITONEAL injection of a large volume of isotonic glucose solution was used by Schecter and coworkers (1) and Darrow and Yannet (2) in a study of the distribution of electrolytes in the various body fluid compartments. This dilution of the ascitic fluid leads to a rapid transfer of electrolytes contained in the extracellular fluid into the peritoneal cavity, followed by a transfer of water into the intracellular compartment to restore osmotic equilibrium between it and the extracellular compartment. Since the rate at which this electrolyte is shifted is greater than that at which glucose is absorbed from the peritoneal cavity, there is a temporary but severe concentration of the extracellular fluid volume. The plasma must act as the intermediary in this transfer, yielding electrolyte and water from itself to the ascitic fluid, replacing it in turn from the various parts of the interstitial fluid, so that there is always a tendency for a disproportionate decline in plasma volume.

As shown first by Swingle and associates (3), the adrenalectomized animal is extremely sensitive to such a dehydration stress. Glucose injections have since been used by many workers (4–10) in studies on the influence of the adrenal cortex on fluid and electrolyte distribution, and on the ability of the adrenalectomized animal to withstand vascular stress. A quantitative comparison of the efficacy of the various adrenal cortical hormones in protecting the adrenalectomized animal against the effects of intraperitoneal glucose injections has not been made, however. It has been shown, in the adrenalectomized dog, that both adrenal cortical extract and the synthetic steroid desoxycorticosterone acetate can protect against fatal circulatory collapse (11). The synthetic hormone loses its protective influence when the animal has been deprived of the adrenal steroids having an oxygen at C-11, such as are present in the whole cortical extract, for some days prior to the experiment (12). The possibility remains that desoxycorticosterone acetate exerts its influence by mechanisms which are qualitatively dissimilar to those which follow therapy with whole cortical extract. The present experiment was undertaken in the hope that a study of fluid and electrolyte transfer following glucose injections could offer a clue to the nature of this difference in action between desoxycorticosterone acetate and cortical extract.

METHODS

Adult male and female rats of approximately 200 gm. in body weight were used. No consistent sex difference in the response has been observed. Adrenalectomy was performed 18 hours before the experiment, and food, but not water, was withdrawn for this interval. Ten cc. per 100 gm. in body weight of a 5.5 per cent glucose solution, warmed to body temperature, was injected intraperitoneally. Hemoglobin de-

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terminations were made on tail blood, by the Newcomer method, before the injection and at 3-hour intervals thereafter. The animals were placed in metabolism cages for urine collection. Ascitic fluid was removed by free drainage through an inch-long slit made in the ventral abdominal wall. Concentrations of chloride in the ascitic fluid were determined by the method of Saifer and Kornblum (13), the specific gravity by a Fischer falling drop apparatus.

Cortical extract[2] was injected in 4 doses as follows: 25 per cent of the total amount subcutaneously at the time of the adrenalectomy, 50 per cent intramuscularly 2 hours before the experiment, and 25 per cent intramuscularly at the time of the glucose injection. The hormones in oil[3] were injected intramuscularly at the time of the operation.

RESULTS

In a previous work (10) it was shown that in the normal intact rat, the period of rapid blood dehydration lasts for about 3 hours after the glucose injection, at which time the ascitic fluid has reached a maximum volume. As glucose continues to be absorbed, water is shifted back into the blood stream, and the concentration of the plasma and interstitial fluids is proportionately reduced. Excretion of urine of low electrolyte content begins at about the 9th hour. As the extracellular electrolytes are thus concentrated, water which had been shifted into the intracellular compartment during the first hours following the glucose injection, is gradually returned. Eventually there is a complete restroation of normal fluid distribution.

The response of the adrenalectomized rat differs in several respects. First, the total amount of electrolyte and water shifted into the peritoneal cavity is always less, yet the serum electrolyte concentrations actually fall lower than in the intact rat. Since this difference cannot be attributed to a faster rate of glucose absorption, the conclusion seems obvious that the ability to shift water and electrolyte from the interstitial reservoirs of the body into the plasma has been impaired. Second, when fluid is returned from the peritoneal cavity, the concentration of the blood is not reduced, but actually is gradually increased until the death of the animal after 4 to 8 hours. The kidneys do not resume normal function in this interval.

A hormone therapy which would entirely restore the resistance of the adrenalectomized rat to the dehydration which follows intraperitoneal glucose injections should *a*) allow a more rapid transfer of electrolyte and water from the interstitial fluid to the peritoneal cavity; *b*) reduce the severity of the initial blood concentration, and allow a later re-dilution with water returned from the peritoneal cavity; *c*) allow a resumption of normal kidney function; and *d*) prevent death.

Fluid and electrolyte shifts into the peritoneal cavity. The total volume and electrolyte content of the ascitic fluid can be used as a measure of the ease with which the animal can transfer electrolyte from the interstitial fluids through the plasma into the fluid of the peritoneal cavity. It was unexpected to find that the volume and electrolyte content of the ascitic fluid of the adrenalectomized rat could not be increased to that characteristic for the intact rat by any of the cortical hormones used in this experiment (table 1). As much as 10 mg. of desoxycorticosterone acetate did not produce a significant change in the volume of the ascitic fluid. Cortical extract increased the volume about a third toward the normal level, but the increment was probably due to the salt contained in the extract.

The smaller volume of ascitic fluid in the adrenalectomized rat is, however, related to the lack of the adrenals. A sham operation, in which both glands were ex-

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[2] The cortical extract was generously supplied by Dr. W. W. Swingle.

[3] The desoxycorticosterone acetate, progesterone, testosterone propionate and estradiol dipropionate were supplied by Dr. E. Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, N. J.

TABLE 1. ASCITIC FLUID CHANGES AT THE END OF THE THIRD HOUR AFTER THE INTRAPERITONEAL INJECTION OF AN ISOTONIC GLUCOSE SOLUTION

Group	No. of Rats	Ascitic Fluid Volume, cc./100 gm. Body Weight	Ascitic Fluid Chloride, m. eq./l.	Ascitic Fluid Chloride, m. eq./ 100 gm. Body Wt.	Ascitic Fluid Protein, gm. per cent	Ascitic Fluid Protein, mg./100 gm. Body Weight
Normal, intact	12	11.7±0.21	85.2	0.997	1.47±0.07	172
Normal, 6 cc. 1% NaCl	6	12.5±0.20	86.2	1.077	1.60±0.09	200
Sham operated	6	11.3±0.09	84.2	0.951	1.55±0.08	175
Unilateral adrenalectomized	6	10.3±0.20	83.4	0.859	1.58±0.09	163
Adrenalectomized:						
Untreated	6	9.0±0.19	85.1	0.766	1.84±0.10	166
1 mg. desoxy. ac.	6	9.5±0.27	83.4	0.792	1.75±0.20	166
3 mg. desoxy. ac.	6	9.0±0.27	80.6	0.725	1.93±0.03	174
5 mg. desoxy. ac.	6	9.2±0.37	82.6	0.760	1.80±0.09	166
10 mg. desoxy ac.	3	8.9	77.1	0.686		
3 mg. desoxy. ac., 6 cc. 1% NaCl	6	10.0±0.30	84.0	0.840		
3 cc. cortical ext.[1]	6	9.7±0.25	86.2	0.836	1.91±0.07	185
6 cc. cortical ext.[1]	6	10.0±0.29	78.8	0.788	1.43±0.16	143
6 cc. 3× cortical ext.[2]	6	10.2±0.20	82.1	0.837	1.60±0.14	163
6 cc. 5× cortical ext.[3]	3	9.7	89.3	0.866	1.75±0.14	170
6 cc. 1% NaCl	6	10.1±0.21	85.1	0.859	1.84±0.18	188
5 mg. progesterone	6	10.2±0.27	84.2	0.859	1.84±0.18	188
5 mg. estradiol dipropionate	6	9.7±0.22	85.8	0.832	1.90±0.10	184
5 mg. testosterone propionate	6	9.6±0.20	81.7	0.784	1.67±0.13	160

[1] Cortical extract, 1 cc. equivalent to 40 gm. of fresh adrenal.
[2] Cortical extract, 1 cc. equivalent to 120 gm. of fresh adrenal.
[3] Cortical extract, 1 cc. equivalent to 200 gm. of fresh adrenal.

posed and manipulated with a degree of trauma greater than that involved in an actual adrenalectomy, resulted in a small, probably insignificant, reduction in the volume of the ascitic fluid. Removal of one adrenal was followed by a reduction in the volume of ascitic fluid to a little less than half-way between the levels characteristic for the intact and the completely adrenalectomized rats. It is interesting that in the hypophysectomized rat, which shows symptoms of a fatal collapse similar to those exhibited by the adrenalectomized rat, the change in volume of the ascitic fluid is the same as that occurring in the intact rat (7, 14).

Changes in the protein concentration of the ascitic fluid have not proved constant enough to serve as a diagnostic sign of the approach of fatal collapse. There was some evidence of an increased protein concentration in adrenalectomized rats either untreated or receiving inadequate hormone therapy. When the total ascitic fluid protein contents are compared, however, this difference largely disappears (table 1).

Changes in the concentration of the blood. The fact that the fluid shifts between extracellular and ascitic fluids is similar in both treated and untreated adrenalectomized rats suggests that the degree of initial blood concentration should be the same in both types of animals. To compare hemoglobin changes in the various groups, the assumption has been made that the concentration of the blood will be strictly proportional to the concentration of the whole of the extracellular fluid, account being taken of the volume of salt solution administered. The actual and theoretical hemoglobin changes are given in table 2. The agreement between the actual and theoretical values is good for both intact and sham-operated rats. Unilaterally adrenalectomized, and all completely adrenalectomized rats, regardless of therapy, showed a greater

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TABLE 2. CHANGES IN HEMOGLOBIN LEVELS AFTER THE INTRAPERITONEAL INJECTION OF AN ISOTONIC GLUCOSE SOLUTION

Group	No. of Rats	Change in Hemoglobin, per cent			
		Estimated Theoretical	3 hr.	6 hr.	9 hr.
Normal intact	6	25.5	23.1	20.7	8.4
Sham operated	6	24.2	23.1	12.4	4.7
Unilateral adrenalectomized	6	20.7	32.0	22.1	14.7
Adrenalectomized:					
Untreated	6	16.3	43.0	44.2	46.0
3 mg. desoxy. ac.	6	16.3	38.4	40.9	30.7
5 mg. desoxy. ac.	6	17.0	37.7	38.0	26.3
10 mg. desoxy. ac.	4	15.9	32.2	25.7	9.6
3 cc. cortical ext.[1]	6	14.6	48.7	36.0	26.8
6 cc. cortical ext.[1]	6	10.3	38.2	54.5	26.3
6 cc. 3× cortical ext.[2]	6	11.1	21.5	15.4	13.6
10 mg. progesterone	3	20.3	20.8	28.2	28.2
10 mg. estradiol benzoate	3	18.2	36.0	30.0	
10 mg. testosterone propionate	3	18.6	33.3	61.8	88.1

[1] Cortical extract, 1 cc. equivalent to 40 gm. of fresh adrenal.
[2] Cortical extract, 1 cc. equivalent to 120 gm. of fresh adrenal.

hemoconcentration than might be expected. This is merely confirmatory evidence for the conclusion that adrenalectomized rats are less able to shift water from the interstitial fluid reservoirs into the blood stream to compensate for the volume lost into the fluid of the peritoneal cavity.

Regardless of the extent of the initial hemoconcentration, if the animal is not entering a state of irreversible circulatory failure, the blood should be diluted by water returned both from the peritoneal cavity and from the intracellular compartment. Reference to table 2 shows that there was no such hemodilution in untreated adrenalectomized rats, in those given saline, or those given sex steroids. Both desoxycorticosterone acetate and cortical extract allowed a hemodilution, with 3 cc. of the latter giving the same order of response as 5 mg. of desoxycorticosterone acetate.

Resumption of kidney function. The intact rat given glucose intraperitoneally resumes normal kidney function when the blood dilution has progressed to the point at which the hemoglobin level is within 6 to 10 per cent of the normal pre-injection level. The untreated adrenalectomized rat, which shows no hemodilution, remains anuric until death. Large amounts of cortical extract are required to allow a resumption of kidney function before the 12th hour (table 3), with the effect of 6 cc. of cortical extract being roughly equivalent to that of 10 mg. of desoxycorticosterone acetate.

Survival period and appearance of circulatory failure. Although, in the untreated adrenalectomized rat, many physical signs point toward the development of a circulatory failure (10), no single criterion is an invariable indicator other than the actual death of the animal. It is difficult to assign a time limit for the experiment. Untreated animals are, almost without exception, dead before the 12th hour. Rats receiving inadequate therapy may show a survival period longer than 12 hours, and yet the experiment must be deemed negative. An experimental period of 24 hours is not entirely suitable, for a small dose of hormone which seems to be protecting the animal for the first 12 hours often will not maintain life for the next 12 hours, when the hormone supply has presumably been depleted.

TABLE 3. URINE PRODUCTION AND SURVIVAL PERIODS OF RATS WITH ASCITIC FLUID REMOVED AT THE END OF THE THIRD HOUR AND WITH ASCITIC FLUID NOT REMOVED

Group	Drained at Third Hour				Undrained Ascitic Fluid						
	No. of rats	Survived, 12 hr.	Survived, 24 hr.	Average survival, hr.	No. of rats	Recovered, 12 hr.	Recovered, 24 hr.	Recovered, 24 hr., %	Survival, av. hr.	Urine volume–12th hr. cc./100 gm. body weight	No .of rats excreting urine 12th hr.
Normal intact	6	6	6		6	6	6	100		2.9	5
Sham operated	6	6	6		6	6	6	100		4.1	6
Unilateral adrenalectomized	6	6	4		6	6	6	100		0.9	3
Adrenalectomized:											
Untreated	6	0	0	6.3	6	0	0	0	6.5	0	0
6 cc. of 1% NaCl	6	0	0	7.1	6	0	0	0	6.2	0	0
1 mg. desoxy. ac.	6	0	0	6.8							
3 mg. desoxy. ac.	6	3	2		6	2	1	17		0	0
5 mg. desoxy. ac.	6	5	4		6	3	1	17		0	0
10 mg. desoxy. ac.	3	2	1		4	4	4	100		0.1	1
2 cc. cort. ext.[1]					6	2	0	0		0	0
3 cc. cort. ext.[1]	6	4	2		6	6	4	67		0	0
6 cc. cort. ext.[1]	6	6	4		6	6	6	100		1.8	3
5 mg. progest.	6	0	0	8.4							
10 mg. progest.					3	2	2	67		0	0
5 mg. testos. prop.	6	0	0	7.3							
10 mg. testos. prop.					3	1	1	33		0	0
5 mg. estrad. diprop.	6	0	0	4.2							
10 mg. estrad. diprop.					3	0	0	0	4.9	0	0

[1] Cortical extract, 1 cc. equivalent to 40 gm. of fresh adrenal.

It is well established that circulatory failure will develop in the adrenalectomized animal after intraperitoneal glucose injections whether the ascitic fluid is removed early in the experiment, or allowed to remain in the body throughout the period of study (3, 7, 10). In the intact rat, recovery is more rapid when the ascitic fluid is not removed and is available for the dilution of the blood and the interstitial fluids (table 3). The same is true for adrenalectomized rats receiving cortical extract therapy; 6 cc. is required for a survival of 12 hours if the ascitic fluid has been removed; 3 cc. will afford the same protection if the ascitic fluid is left in the body. The difference is not clearly discernible with desoxycorticosterone acetate therapy. The data in table 3 show that 6 cc. of cortical extract afforded a degree of protection for 24 hours similar to that obtained with 10 mg. of desoxycorticosterone acetate in test animals from which the ascitic fluid was not removed. Testosterone and progesterone in large doses may have afforded a partial protection. Estradiol actually shortened the survival period.

DISCUSSION

Both whole cortical extract and desoxycorticosterone acetate will allow the redilution of the blood, the resumption of renal activity, and the maintenance of life in the adrenalectomized rat given an intraperitoneal injection of an isotonic glucose solution. Neither material can alter the decreased ability to transfer electrolyte and water from the various regions of the extracellular fluid to the peritoneal cavity. Two factors, which do not seem to be wholly interdependent, must be recognized in the response of the adrenalectomized rat to glucose injections. One has to do with the development of a progressive circulatory failure leading to death of the animal. The other is concerned with the ease with which electrolyte and fluid can be interchanged between plasma and interstitial fluid. The possibility has not been eliminated that the adrenal medulla might be involved in this latter phase of the response. The mechanisms underlying the two factors are not understood.

As far as the prevention of fatal collapse is concerned, 6 cc. of cortical extract seems to be as effective as 10 mg. of desoxycorticosterone acetate. The daily minimal

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maintenance requirement of the young adrenalectomized rat is of the order of 0.25 cc. of the extract used and 0.05 mg. of desoxycorticosterone acetate. Hence it required roughly 24 times the maintenance dose of extract and about 200 times that of desoxycorticosterone acetate to prevent fatal collapse in this experiment (14). This ratio of 1:9 is somewhat of the order of that observed by Eversole, Gaunt and Kendall (15) who used the rate of excretion of excessive amounts of orally administered water as a functional test for adrenal insufficiency. In their study, the beneficial effect of whole cortical extract was presumably due to the carbohydrate-active hormones. The same assumption could be applied to the results obtained in this study.

The weak protective action that desoxycorticosterone acetate exerts in maintaining life after intraperitoneal glucose injections does not appear to be related to a specific effect upon the kidney nor upon the internal distribution of body fluids. It did not increase the total amount of extracellular fluid available for dilution by the injected solution; its action in allowing a re-dilution of the blood was weaker than that of extract; it did not shorten the interval required for the resumption of normal kidney function, nor did it allow a more rapid excretion of urine. Hence no evidence has been found for an action of desoxycorticosterone qualitatively different from that of cortical extract.

SUMMARY

The ability of desoxycorticosterone acetate to protect the adrenalectomized rat against the fatal collapse which follows an intraperitoneal injection of an isotonic glucose solution has been compared with that of whole adrenal cortical extract and the sex hormones progesterone, testosterone and estradiol. The decreased ability of the adrenalectomized rat to transfer water and electrolyte from the interstitial spaces into the peritoneal cavity, via the blood stream, could not be corrected to that of the intact rat by any therapy used. Likewise the initial hemoconcentration in the period of rapid water transfer to the peritoneal cavity could not be reduced to that characteristic of the intact rat. In its ability to allow a later hemodilution, to restore urine flow, and to prevent the symptoms of a fatal collapse, desoxycorticosterone acetate proved about 9 times weaker than cortical extract. As judged by the criteria employed in this experiment, there was no evidence that the action of desoxycorticosterone acetate was qualitatively different from that of cortical extract.

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EFFECT OF HISTAMINE UPON CAPILLARY PERMEABILITY IN THE SKIN AND MUSCLE OF NORMAL AND ADRENALECTOMIZED RATS[1]

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IT IS WELL KNOWN that the resistance of the rat and other species to fatal histamine shock is markedly reduced by adrenalectomy (1, 2), and that treatment with adrenal cortical hormone will restore this resistance to normal (3). The possibility arises that the sensitivity of the adrenalectomized rat is due to an enhanced action of histamine in increasing capillary permeability. This situation would result if either the threshold of capillary sensitivity to histamine or the rate of histamine inactivation is decreased following adrenalectomy.

To obtain information on this point, studies were undertaken to determine *a*) the minimal concentration of histamine which would increase capillary permeability and *b*) the duration of activity of histamine on capillary permeability in the skin and muscle of normal and adrenalectomized rats.

METHODS AND RESULTS

Changes in capillary permeability[2] induced by histamine were evaluated by comparing the amounts of trypan blue accumulated in histamine-treated areas of skin with those in control areas. This was done on the basis that abnormally permeable capillaries allow escape of dye at an increased rate and thus excessive accumulations of dye in the tissue occur (4). In utilizing dye methods for evaluating changes in capillary permeability, the question arises as to how closely dye diffusion reflects the passage of plasma protein through capillaries.

The finding that dyes related to trypan blue combine with plasma proteins (6), suggested that trypan blue might likewise be bound to plasma proteins. In this case, passage of trypan blue through capillaries might, in effect, represent the diffusion of a trypan blue-plasma protein complex. The following experiments were therefore performed to determine whether or not trypan blue combines with plasma proteins.[3] *a*) Dialysis of trypan blue solutions (National Aniline and Chemical Co., C.I. No. 477 Lot No. 7286) at pH 7.6 revealed that in the absence of plasma, dye came through a regenerated cellulose membrane slowly, while in the presence of plasma, no detectable diffusion of dye was noted after a dialysis lasting two days. *b*) Electrophoretic studies with the use of the Tiselius apparatus demonstrated that at pH 7.6 trypan blue in the presence of plasma migrates partly with the albumin fraction, while the bulk moves somewhat less rapidly than the α-globulin. Trypan blue in salt solution at this pH, has a higher mobility than the albumin fraction in plasma, although the dye does not appear to be entirely uniform in its rate of migration. The charge on the dye is negative, as is the charge on the plasma proteins at this pH.

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[2] The term capillary permeability, as discussed in a previous paper (5), refers to the passage of large colloidal-sized particles of the order of plasma protein, through the capillary membrane.

[3] Grateful acknowledgement is made for the aid of Dr. John Mehl and Edward Sexton, University of Southern California Medical School, in these experiments.

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The electrophoretic data demonstrate that the mobility of trypan blue is decreased by the addition of plasma to that of the plasma proteins. This seems best explained on the basis that dye combines with plasma proteins. The results of dialysis experiments also fit with this interpretation. It therefore appears, by virtue of the demonstrated combination of trypan blue with plasma protein, that trypan blue may be an accurate indicator for capillary permeability to plasma protein.

The rats used in these experiments, weighing between 180 and 220 gm., received a diet of Purina fox chow. The bilaterally adrenalectomized rats were operated in the usual manner, 5 to 7 days prior to the experiment, after which time histamine sensitivity is known to be well established (1). During the postoperative period the rats were maintained in good health by substituting saline for drinking water. As is known, such treatment of adrenalectomized rats with saline does not remove the marked sensitivity to histamine. Finally, the completeness of the adrenalectomies was checked at the termination of the experiment.

For study of capillary permeability in skin, the abdomens of the rats were carefully shaved one day before the experiment. Rats were injected intravenously, through the tail vein, with 0.4 to 0.6 cc. per 100 gm. of body weight of a 1 per cent trypan blue solution in saline. Histamine was applied to the skin by placing drops of histamine solution over an abdominal area which had been stabbed a few seconds earlier with a fine 27-gauge hypodermic needle. The solution was allowed to cover the abdominal stab for 5 minutes, after which time the solution was absorbed with cotton, its removal being accomplished without touching the skin. Other areas were similarly treated with saline solution and served as controls.

Using this technic of administering the test solution through the skin opening provided by the hypodermic needle, it was observed that saline produced minimal dye accumulation just at the stab point, independent of whether the saline was administered shortly before or after intravenous dye injection. This finding contrasts with the fact of marked dye accumulation which results following intracutaneous injections of saline at these time intervals. A positive histamine reaction with the stab technic is evidenced by a definite ring of dye, concentrated around the stab point.

Freshly prepared solutions of histamine acid phosphate[4] in 0.9 per cent NaCl, containing 1.0, 2.0, 3.3, 5.0, and 10.0 mg. per cc., respectively, were used.

Effect of Histamine upon Capillary Permeability in the Skin of Normal and Adrenalectomized Rats

Capillary sensitivity. Normal and adrenalectomized rats were intravenously injected with trypan blue and immediately thereafter, histamine solutions of various concentrations and saline were administered to skin capillaries through the stab wounds. Table 1 illustrates the results obtained. It will be seen that the minimal effective concentration of histamine in both groups is approximately the same, no significant differences being noted. While this method does not allow determination of absolute values, it appears that the skin capillaries of adrenalectomized rats are not hypersensitive to the action of histamine on capillary permeability.

Duration of action. The duration of histamine action was ascertained by administering a fixed maximal dose of histamine at various time intervals prior to dye injection, and determining the point at which a positive response was no longer obtained. Various areas of the abdominal skin of normal and adrenalectomized rats were treated with a solution containing 10 mg. per cc. of histamine acid phosphate in saline, and with saline alone at 40, 30, 20, 15, 10, and 5 minutes prior to dye injection. A saline-treated area served as a control for the areas treated with histamine at each time interval. The

[4] Histamine acid phosphate was supplied by Burroughs, Wellcome, & Co., Inc., New York City.

TABLE 1. MINIMAL EFFECTIVE CONCENTRATION OF HISTAMINE UPON CAPILLARY PERMEABILITY IN SKIN

Histamine mg./cc.	Normal Rats				Adrenalectomized Rats			
	No. of Observations	+	?	−	No. of Observations	+	?	−
1.0	10	0	1	9	10	0	3	7
2.0	10	2	3	5	10	3	2	5
3.3	10	10	0	0	10	9	1	0
5.0	10	10	0	0	10	10	0	0
10.0	10	10	0	0	10	10	0	0

results obtained are shown in table 2, in which it will be seen that only slight differences in the duration of histamine action were obtained between normal and adrenalectomized rats. It is of interest that the action of histamine in skin is of a relatively transient duration, the increased capillary permeability being reversed in approximately 15 minutes. The differences shown in table 2 are not large, and larger groups of animals would be necessary to determine the significance of this difference. In any case, it is clear that the difference between the normal and adrenalectomized animals is less than 5 minutes, which would amount at most, to a 1.5-fold increase of histamine action in skin following adrenalectomy.

Effect of changes in cutaneous blood flow upon dye accumulation. The results obtained in the previous section may be subject to criticism on the ground that blood flow in skin is decreased following adrenalectomy, and that, consequently, dye accumulation would tend to be reduced in the adrenalectomized rats. Experiments were therefore undertaken to investigate the influence of impaired blood flow upon dye accumulation.[5] Four rats, while anesthetized with ether, were placed on their backs and a ridge of lanolin was applied ventrally, on the midline, so that the abdomens were halved longitudinally. One side of the abdomen was warmed by continually applying strips of warm gauze soaked in water at 45°C., while the other side was cooled by applications of cold gauze, soaked in ice water. The lanolin ridge effectively prevented the wet packs from transferring fluid to the opposite side. After a few minutes, 0.2 cc. of a saline solution containing 10 mg. per cc. of histamine acid phosphate was injected intracutaneously into areas of both sides of the abdomen, and the hot and cold applications to the appropriate sides were continued. After 10 minutes, dye was injected intravenously. The applications were continued and dye accumulation noted in both sides. The following findings were observed in all instances. *a*) The coloration of the skin in the cooled area was less than that observed in the warmed area. *b*) Excessive dye accumulation at the site of histamine injection was evident on both sides within a few minutes. *c*) Although the amount of dye concentrated in the histamine-treated area on the warmed side was initially

TABLE 2. DURATION OF HISTAMINE ACTION

Time Before Dye Injection, min.	Normal Rats				Adrenalectomized Rats			
	No. of Observations	+	?	−	No. of Observations	+	?	−
40	3	0	0	3	4	0	0	4
30	3	0	0	3	4	0	0	4
20	8	0	3	5	9	1	2	6
15	8	3	5	0	9	5	4	0
10	8	8	0	0	9	9	0	0
5	8	8	0	0	9	9	0	0

[5] Thanks are due Dr. Myron Prinzmetal for helpful suggestions in this section.

greater, within 10 minutes both histamine-injected areas had attained approximately the same degree of coloration. These findings indicate that although the decrease in blood flow induced by cold does reduce the small amount of dye concentrated by skin with normal capillary porosity, decreased blood flow does not prevent the development of excessive dye accumulation in areas with abnormally permeable capillaries. It further appears that, in time, areas with decreased blood flow, but with porous capillaries, can concentrate as much dye as those areas with porous capillaries and with normal or increased blood flow.

These findings are in full agreement with a previous theoretical analysis of the effect of blood flow upon dye accumulation in skin (5). It therefore appears that possible changes in blood flow induced by adrenalectomy are without serious influence upon the results obtained in the previous sections.

Effect of Histamine upon Capillary Permeability in the Muscle of Normal and Adrenalectomized Rats

Ten normal and an equal number of adrenalectomized rats were injected intravenously with dye. Immediately thereafter, 0.2 cc. of a solution containing 10 mg. per cc. of histamine was injected directly into the belly of one gastrocnemius muscle, and an equivalent volume of saline was injected similarly into the other gastrocnemius as a control. Twenty to 30 minutes later, the muscles were examined and compared. In no instance, in either the saline or histamine-injected muscles of the normal or adrenalectomized animals, was significant dye concentration observed. In 4 instances, a histamine solution containing 10 mg. per cc. injected directly into the exposed gastrocnemius muscle with intact blood supply, likewise failed to produce dye concentration. Further evidence that the muscle capillaries are qualitatively different from skin capillaries was apparent from the finding that xylol applied directly to the gastrocnemius muscle fibers likewise failed to produce trypan-blue accumulation. In the fascia of muscle, however, xylol did induce dye concentration as it does in skin. At the same time that these negative results were obtained in muscle with histamine and xylol, dye accumulation was obtained in skin with these agents.

Previous observations that muscle accumulates excessive amounts of dye after severe trauma (5), or after the injection of large amounts of dye (unpublished results) serve to demonstrate that muscle has the ability to fix dye. The fact that histamine and xylol fail to cause dye accumulation in the gastrocnemius muscle cannot be explained on the basis that skin, but not muscle, has an affinity for trypan blue. It therefore appears that histamine and xylol are without effect upon capillary permeability in muscle.

DISCUSSION

The results indicate that the skin and muscle capillaries of adrenalectomized rats are not hypersensitive to the activity of histamine in modifying capillary permeability. Although there may be a decreased rate of histamine inactivation in skin following adrenalectomy, the duration of histamine activity would at the most be increased only 1.5 times. It is clear that this possible increased interval of histamine action in skin cannot quantitatively account for the approximately 20-fold (1) increase in histamine sensitivity which follows adrenalectomy. It thus appears that the sensitivity of adrenalectomized rats to histamine is not explicable in terms of an enhanced action of histamine in increasing general capillary permeability.

This report does not include a study of the possibility that the capillaries of some specific vital organ following adrenalectomy, are sensitized to histamine by changes in capillary permeability, and thus is responsible for the fatal histamine sensitivity.

However, it would seem to be difficult to explain the rapid deaths which occur within the first hour following histamine injection into adrenalectomized rats (1, 2), on the basis of an edema produced in this hypothetical vital organ by histamine action on capillary permeability. It would appear that the low degree of resistance of the adrenalectomized rat to histamine is due to factors other than sensitivity to changes in capillary permeability.

Although the nature of the hypersensitivity to histamine is as yet unknown, there are explanations which merit consideration. The finding (7) that the rate of histamine removal from certain tissues is lowered after adrenalectomy suggests that the adrenalectomized rat is hypersensitive because the rate of histamine inactivation is decreased. Although this difficulty in destroying histamine may indeed be part of the explanation, the differences reported for the rates of histamine disappearance in normal and adrenalectomized rats cannot successfully account for a 20-fold increase in histamine sensitivity.

There has been a considerable body of evidence which indicates that the low degree of resistance of adrenalectomized animals to various types of stress (hemorrhage and muscle trauma) is related to a difficulty in the transfer of extravascular fluid to the vascular system, in response to a blood volume deficiency (8). It seems likely that the relative inability of the adrenalectomized rat to compensate for the reduction in circulating blood volume induced by histamine through both capillary permeability and dilatation, may well be a large part of the explanation for the histamine sensitivity of adrenalectomized animals.

It is of considerable interest to note that histamine and xylol, both of which increase capillary permeability in skin, are without such action in gastrocnemius muscle. This observation is another instance illustrating the qualitative differences in reactivity of various capillary systems to the same stimulus, such as has been reported previously with hormonal steroids (4). This observation serves to demonstrate that conclusions regarding generalized capillary permeability derived from the study of a single capillary system are not necessarily valid. Since much of the work in this field has been performed using the capillaries of single tissues, such as skin, mesentery and frog web, it is clear that many findings in the field of capillary permeability warrant critical examination as regards their generalized application.

SUMMARY

The effect of histamine upon capillary permeability in the skin and muscle of normal and adrenalectomized rats has been studied. The skin capillaries of adrenalectomized rats are not hypersensitive to the action of histamine on capillary permeability, nor is the action of histamine in skin increased more than 1.5 times. Histamine (and, xylol), however, was found to be without effects upon capillary permeability in the gastrocnemius muscles of normal and adrenalectomized rats. The relationship of these findings to the question of the hypersensitivity of adrenalectomized rats to histamine has been discussed.

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ASCORBIC ACID-GONADOTROPIC HORMONE RELATIONSHIPS IN THE CHICK[1]

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DURING the past decade evidence has been accumulating that ascorbic acid may play a part in the regulation of the normal reproductive processes and that the utilization of vitamin C may be partially under endocrine control. Biskind and Glick (1) found that the corpus luteum of pregnancy contained twice as much vitamin C as that of the non-pregnant guinea pig. Ley (2) reported that ascorbic acid was of therapeutic value in the treatment of habitual abortion of women. Phillips et al. (3, 4) found that the subcutaneous injection of ascorbic acid was of value in the restoration of fertility in certain impotent bulls and that similar treatment of cows was followed by the correction of some types of sterility.

Mosonyi (5) observed a reduction in the ascorbic acid content of the liver and adrenals following the injection of either the male or female sex hormones; Winkler and Binder (6) demonstrated a decrease in pituitary ascorbic acid following the injection of chorionic gonadotropin; and Israel and Meranze (7) reported that ascorbic acid has a progesterone-like effect on the endometrium of the ovariectomized mouse, rat and rabbit. Erb and Andrews (8) produced decreases in blood plasma-ascorbic acid of up to 67 per cent within 24 hours in both bulls and cows by the injection of pregnant mare serum gonadotropin. Andrews and Erb (9) observed that the injection of 1000 and 2000 R.U. of pregnant mare serum gonadotropin was followed by decreases in venous blood plasma-ascorbic acid of 42 and 56 per cent, respectively, within a period of 5 hours in the castrated bovine.

Giedosz, as reported by Di Cio and Schteingart (10), injected adult rabbits with gonadotropic hormone and simultaneously with gonadotropin and ascorbic acid. The ovaries of the does injected with both ascorbic acid and gonadotropin were larger than those of the animals receiving only the gonadotropic hormone. Di Cio and Schteingart (10) injected 2 to 4-month-old male and female rats with 25 R.U. of gonadotropin alone, 50 mg. of ascorbic acid alone, and 25 R.U. of gonadotropin plus 50 mg. of ascorbic acid, daily for 20 to 30 days. They found that ascorbic acid alone had no effect on gonad weight, but that gonad size was almost doubled by the addition of ascorbic acid to gonadotropin as compared with the hormone alone. Di Cio and Schteingart (10) could not demonstrate an increase in the ovarian weight of rabbits injected with both ascorbic acid and gonadotropic hormone as described by Giedosz. They reported that no augmentation was observed in the rat following short periods of treatment (3 days) and that the combination of ascorbic acid and gonadotropin produced no apparent augmentation in adult rats.

If vitamin C has a rôle in the regulation of the normal reproductive processes, and if the injection of gonadotropin brings about a reduction in blood plasma-ascorbic acid, the frequent failure of the gonadotropic hormone to correct lowered fertility may be at least partially explained.

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The data which follow are a part of a study of the endocrine regulation of ascorbic acid metabolism. The experiments were conducted for the purpose of determining whether or not pregnant mare serum gonadotropin can be augmented by ascorbic acid[2] in the chick.

MATERIALS AND METHODS

Experiment 1. Ninety-nine one-day-old male White Leghorn chicks hatched on Feb. 26, 1942 were divided into 4 groups as shown in table 1. The gonadotropic hormone used was equine pregnancy serum[3] standardized to contain 50 R.U. per cc., and

TABLE 1. EFFECT OF PREGNANT MARE SERUM GONADOTROPIN AND ASCORBIC ACID ON THE TESTIS AND BLOOD PLASMA-ASCORBIC ACID OF THE CHICK

Daily Treatment	No. of Chicks	Length of Treatment	Age of Chicks at Autopsy	Ave. Testis Weight	Ave. Blood Plasma Ascorbic Acid
		days	*days*	*mg.*	*mg. %*
Experiment 1					
Controls	25	5	6	12.4	
50 mg. A.A.[1]	23	5	6	13.5	
10 R.U. G.[2]	26	5	6	29.9	
10 R.U. G.+50 mg. A.A.	25	5	6	26.2	
Experiment 2					
Controls	22	10	12	13.8	.713
10 R.U. G.	16	10	12	45.2	.629
10 R.U. G.+25 mg. A.A.	19	10	12	42.4	1.515
10 R.U. G.+50 mg. A.A.	19	10	12	37.6	1.611
10 R.U. G.+75 mg. A.A.	16	10	12	43.7	1.580
Experiment 3					
Controls	18	10	39	50.7	.499
10 R.U. G.	18	10	39	80.3	.534
10 R.U. G.+50 mg. A.A.	22	10	39	83.9	.836
Experiment 4					
Controls	25	10	12	18.1	.142
10 R.U. G.	26	10	12	46.1	.103
10 R.U. G.+50 mg. A.A.	24	10	12	51.0	.708
10 R.U. G.+75 mg. A.A.	19	10	12	41.0	.803

[1] A.A.=Ascorbic Acid. [2] G.=Gonadotropin.

was injected subcutaneously. Crystalline ascorbic acid was made up fresh daily in a phosphate buffer in the proportions of 50 mg. of ascorbic acid in 0.2 cc. of solution. The solution had a pH of 2.9 and was subcutaneously injected at a site other than that in which the gonadotropin was administered. Injections were begun on Feb. 27, 1942, and continued daily for 5 days. The chicks were killed on the day following the last injection and the testes were removed and immediately weighed on a precision balance.

Experiment 2. Ninety-two 2-day-old male White Leghorn chicks hatched on March 9, 1942 were divided into 5 groups and treated as shown in table 1. Since the ascorbic acid solution used at pH 2.9 in the previous experiment tended to cause some irritation at the injection site, it was dissolved in 1 N NaOH in the proportions of 50 mg. of ascorbic acid in 0.2 cc. of solution and its pH was 4.5. The chicks were autopsied on the day following the last injection. The testes were removed and

[2] The ascorbic acid was furnished by Merck and Co., Rahway, N. J.

[3] The equine gonadotropin (Gonadin) was furnished by Donald H. Wonder, Director of the Endocrine Division, Cutter Laboratories, Berkeley, Calif.

weighed as previously described and in addition the blood from groups of 5 chicks each was pooled and plasma ascorbic acid determined photometrically by the method of Mindlin and Butler (11).

Experiment 3. In order to determine the influence of age on the ascorbic acid-gonadotropin response, 59 White Leghorn male chicks hatched on March 30, 1942 were allowed to reach the age of 29 days before treatment was begun. The chicks were divided into 3 groups and treated as shown in table 1. The crystalline ascorbic acid was made up in solution as described in *experiment 2*, and subcutaneous injections of both vitamin C and pregnant mare serum gonadotropin were made for 10 consecutive days. The chicks were killed on the day following the last injection, the testes weighed and blood plasma-ascorbic acid determined as previously described.

Experiment 4. Since the testes of the chicks which received 50 mg. of ascorbic acid daily in conjunction with gonadotropin in *experiment 2* tended to be smaller than those of the chicks which received 25 and 75 mg. of ascorbic acid, it was decided to repeat this experiment in part. Ninety-four 2-day-old chicks hatched May 11, 1942 were divided into 4 groups treated as shown in table 1. The gonadotropic hormone and ascorbic acid were administered as in *experiments 2* and 3. Treatment was begun on May 13, 1942, terminated 10 days later and the chicks killed on May 23. Testis weights and plasma ascorbic acid values were obtained.

The data on the testis weight for each experiment were analyzed[4] for variance according to the methods described by Snedecor (12).

RESULTS

Ascorbic acid alone, when administered at the rate of 50 mg. daily for 5 days, had no significant effect on testis weight as shown in *experiment 1*. The testes of the chicks which received pregnant mare serum gonadotropin, either alone or in combination with varying levels of ascorbic acid, were significantly heavier than those of the controls and the percentage increases ranged from 158 in *experiment 3* to 316 per cent in *experiment 2*. The addition of ascorbic acid to gonadotropin produced no significant changes in testis weight. Although there appeared to be a slight depression in testis weight in the group receiving 10 R.U. of gonadotropin plus 50 mg. of ascorbic acid in *experiment 2*, statistical analysis for variance showed the difference to be of doubtful significance and this was verified by repetition in *experiment 4*.

The primary objective of the present investigation was to determine whether or not the injection of ascorbic acid simultaneously with gonadotropin would increase the effectiveness of the gonadotropic hormone. The blood plasma-ascorbic acid levels obtained in *experiments 2*, 3 and 4 are presented for supplementary rather than primary value. The data on the results with vitamin C did not lend themselves to statistical analysis, but several trends are worthy of notice. The injection of gonadotropin in the chick, under the conditions described, did not bring about a reduction in plasma-ascorbic acid as the authors previously found in cattle (8, 9). There was a tendency for lowered blood values to follow treatment with pregnant mare serum gonadotropin alone in *experiments 2* and 4, but a slight increase in *experiment 3*. The differences between the ascorbic acid values in the 3 experiments were marked. The controls in *experiment 2* had over 500 per cent more blood-vitamin C than the corresponding group in *experiment 4*. The chicks in all studies were of the same breed, from the same source, fed the same ration and managed similarly. The fact that the testis weights of similar groups in *experiments 2* and 4 were not significantly different

[4] We are indebted to Dr. Wayne A. Anderson, of Purdue University, for the statistical treatment of the data.

is further indication of the homogeneity of the animals, at least from the standpoint of gonad response. Two variables were introduced, age in *experiment* 3 and season in *experiments* 2, 3 and 4. These factors could easily influence vitamin C metabolism, although the possible variation of the ascorbic acid content of the eggs from which the chicks were hatched should not be overlooked. The ascorbic acid phase of the study is being continued and the findings will be presented subsequently.

DISCUSSION

The male White Leghorn chick was selected for this study because of the responsiveness of the testes to gonadotropic hormone. The increase in testis weight which followed the injection of pregnant mare serum gonadotropin alone or in combination with ascorbic acid was consistent, but ascorbic acid, under the conditions administered, had no augmentative effects. It is possible that species and age differences exist. In the present experiment ascorbic acid in combination with gonadotropin had no more effect on 2 to 12-day-old chicks than on 28 to 39-day-old animals, and the trends following a 10-day-injection period were not significantly different from those following a 5-day interval of treatment. The injection of 2 to 4-month-old rats for periods of 20 to 30 days (10) is strikingly different from the most commonly employed techniques of gonadotropic hormone assay. Although the quantitative measurement of gonadotropins may be arrived at by many different assay methods, immature rats ranging from 21 to 24 days of age are very often injected daily for 3 to 5 days and autopsied on the day following the last injection of test substance. Injection periods of 20 to 30 days have not been used because of the time involved. Andrews (13) and others report extensive spermatogenesis and the formation of mature spermatozoa in rats 60 to 70 days of age. It is interesting to observe that Di Cio and Schteingart (10) find male rats of 2 to 4 months of age most responsive to ascorbic acid-gonadotropin treatment, and that 'mature' rats cannot be similarly affected. Although rats must be considered 'sexually mature' when large quantities of spermatozoa are being produced (at 2 to 4 months) it may be that the onset of spermatogenesis is accompanied by physiologic conditions which sensitize the testis to gonadotropin-ascorbic acid combinations at this particular time. Studies which are being made in this laboratory on the effects of pregnant mare serum gonadotropin and ascorbic acid on the weight of the ovaries and testes of 24 to 28-day-old rats indicate, at the moment, that ascorbic acid has no augmentative effects on rats of this age.

The relationship of blood-ascorbic acid levels and testis weight is of interest, although of unknown significance at present. It has been generally agreed by the nutritionists that extraneous sources of vitamin C are unnecessary in the fowl, and indeed, in species other than man, monkey and the guinea pig. The blood values for ascorbic acid in the control chicks were more than 500 per cent greater in March than for a corresponding group in May. It is also worthy of note that the injection of 50 mg. of ascorbic acid daily for 10 days resulted in an increase of plasma-vitamin C of approximately 200 per cent in March, in contrast to over 500 per cent produced by the same treatment in May. Although the experiment was not designed to measure the saturation point of fowl blood for ascorbic acid, it would appear that a content of 1.5 to 1.6 mg. per cent is nearly maximum in chicks of the ages studied. In spite of the marked differences in blood-ascorbic acid content, the testis weights were not significantly different.

SUMMARY

The injection of ascorbic acid alone had no influence on the testis weight of the White Leghorn chick, and ascorbic acid injected in combination with pregnant mare

serum gonadotropin did not augment the action of the gonadotropic hormone. There was no apparent relationship between blood plasma-ascorbic acid and the testis weight.

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INFLUENCE OF ADRENAL PREPARATIONS ON FISH MELANOPHORES[1]

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THERE HAS LONG been a need for a simple method for assaying adrenal cortical extracts. Santa and his associates (1) have reported that epinephrin-free adrenal cortical extracts, as well as desoxycorticosterone, cause contraction of the melanophores of the scales of the fish *Cyprinus carpio*. This contraction, because it was incomplete and slow, could be distinguished from that caused by epinephrin, which was complete and rapid. The specificity claimed would be an improvement over the time consuming, expensive methods now in use. Therefore, we have attempted to employ the technique on preparations which had been assayed by other methods. We failed to find in this method either a quantitative or qualitative test for adrenal cortical substances.

METHODS AND RESULTS

The scales of various species of fish were tested in an effort to find one in which melanophore distribution and reaction were suitable. Scales of *Barbus conchonius* (Rosy Barb), *Brachydanio rerio* (Zebra fish), *Lebistes reticulatus* (Guppy), *Etroplus maculatus* (Orange Chromide), and *Carassius auratus* (Goldfish) were used.

The scales were taken from the dorso-lateral portion near the tail of the fish and were kept in Ringer's solution (NaCl 0.7%, KCl 0.03%, $CaCl_2$ 0.026%, $NaHCO_3$ 0.003%) of pH 7.4 until tested. In this solution the pigment cells remained dilated indefinitely. In the course of the experiments the scales were transferred to the test solution contained in a hanging-drop slide, in which they were studied under the microscope with low magnification.

The Orange Chromide was found to be most suitable, since its pigment cells are small, of uniform size, cover the whole melanophore-bearing region of the scale, and are very sensitive to epinephrin. The melanophores in the scales of the young Goldfish (black) are also satisfactory. In the majority of cases the pigment cells of scales taken from these two species were expanded, while melanophores in the scales removed from the Rosy Barb were more often in a state of contraction. Also, in the case of the Rosy Barb, pigment cells initially thought to be expanded were found to expand still further when placed in adrenal extracts. Scales from the Guppy were very small and difficult to handle.

The following stock solutions were used for testing, suitable dilutions being made as indicated:

Unfractionated adrenal extract

Lot I. 4 cat units (2) per cc. No epinephrin
Lot II. 16 cat units per cc. Epinephrin not more than 1:1,000,000

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[1] Aided by grants from the National Research Council Committee on Research in Endocrinology and the Comly Fund of the Ohio State University.

Cortin fraction
4 cat units per cc. Epinephrin content 1:500,000
Sublimed cortin fraction
16 cat units per cc. No epinephrin
Sodium factor fraction
Lot I. 4.0 units (3) per cc. No epinephrin
Lot II. 2.8 dog units per cc. No epinephrin
Lot III. 2.8 dog units per cc. No epinephrin
Desoxycorticosterone-saturated solution (0.8 mg. in 1 cc. of Ringer's solution)

The epinephrin[2] used was adrenalin chloride, $1:10^{-3}$ concentration.

TABLE 1. RESPONSE OF ORANGE CHROMIDE AND GOLDFISH MELANOPHORES

Test Substance	Concentration	Reaction
Adrenalin chloride	$1:10^{-4}$ to $1:5\times10^{-5}$	Complete contraction in 1 to 5 min.; contraction maintained to end of test
	$1:10^{-6}$ and above	Partial contraction in 2 to 45 min.; expansion again before completion of test
Unfractionated adrenal extract	As high as 1:1	Slow, partial contraction within 15 to 25 min.; no further change until end of test
Lot I (4 C.U./cc.)	1:2 and above	No reaction
Lot II (16 C.U./cc.)	Any concentration used	No reaction
Cortin fraction (4 C.U./cc.)	Stock solution to 1:10	Complete contraction of peripheral melanophores in 1 min.; no further change
	Above 1:10	No reaction
Sublimed cortin	Any concentration used	No reaction
Sodium factor fraction		
Lot I (4.0 D.U./cc.)	Stock solution	Complete contraction in 10 to 15 min.; no further change
	Dilutions	No reaction
Lot II (2.8 D.U./cc.)	Any concentration used	No reaction
Lot III (2.8 D.U./cc.)	Any concentration used	No reaction
Desoxycorticosterone	Stock solution to $1:8\times10^{-5}$	Partial contraction in 2 to 15 min.; some cells completely contract after 15 min.; expansion again after one hour
Parathyroid extract	Stock solution	Partial or complete contraction in 20 min.; no further change
	Dilutions	Slow partial contraction in 20 min.; no further change

An aqueous extract of the parathyroid glands[3] of cattle, was tested, since it might serve as a control against the specific reaction said to be caused by adrenal extracts.

Dilution of the test substances was made with Ringer's solution at the time of the immersion of the scales.

The scales immersed in the test solution were observed for a period of 1 to 3 hours. In order to determine whether maximal contraction had been attained, the scales were finally transferred to an epinephrin solution of $1:10^{-5}$ concentration. This induced maximal contraction if it did not already exist. Scales were kept in Ringer's solution as controls.

Scales from 10 individual Goldfish and 10 individual Orange Chromides were tested. At least 50 scales were used to test each dilution of extract.

The results are shown in table 1.

[2] Adrenalin chloride was supplied by Parke, Davis & Co., Detroit, Mich.
[3] The parathyroid extract was supplied by Eli Lilly & Co., Indianapolis, Ind.

DISCUSSION

The melanophores of fish scales do not react uniformly. It appears that those in densely pigmented scales, if they react at all, do so much more slowly than do those in sparsely pigmented scales. The melanophores of neighboring scales may behave differently to the same treatment. The cells of one scale may become completely contracted while those of another are only partially contracted.

Reactions to epinephrin depend somewhat upon the dilution. A rapid complete contraction of melanophores occurred within one or two minutes after immersion of the scale in epinephrin solutions of dilutions between $1:10^{-4}$ and $1:10^{-6}$. Dilutions of $1:10^{-6}$ and greater called forth only a slowly-developing, partial contraction. This appeared to be somewhat similar to the slowly-developing, partial contraction caused by adrenal cortical extracts and desoxycorticosterone. Therefore, it seems that the 'all or none' effect does not apply to solutions of epinephrin more dilute than $1:10^{-6}$. Thus, the test does not distinguish cortical preparations and dilute epinephrin solutions.

The fish-scale-melanophore reaction is not an infallible test for adrenal cortical hormones. Our *Lot I Unfractionated Extract* caused a positive reaction in the scales of both the Orange Chromide and the Goldfish, yet it was of low potency in maintaining adrenalectomized cats. On the other hand, *Lot II Unfractionated Extract* was very potent, yet it was without effect on the fish scales. Likewise, a cortin fraction with low potency for maintenance of life caused complete contraction. This may be explained by the small amount of epinephrin present. When this was diluted, a lesser effect was obtained. A sublimed cortin fraction with high potency for life maintenance elicited no melanophore reaction. The sodium factor fraction, *Lot I*, caused complete contraction of melanophores in scales from both the Orange Chromide and the Goldfish, while the sodium factor fraction, *Lots II* and *III* (used only in the case of the Orange Chromide) elicited no changes, yet *Lots II* and *III* were very potent in retaining sodium.

Desoxycorticosterone caused partial contraction of the pigment cells of scales from both the Orange Chromide and the Goldfish. This partial contraction was similar to that described by Santa for desoxycorticosterone and adrenal cortical extracts. The time required for the contraction to develop, however, was found to be much shorter than that described by Santa, for in these experiments the cells had reached the limit of their contraction in from 2 to 15 minutes, whereas Santa reports a slowly-developing contraction over a period of 45 minutes. Some cells in scales from the Orange Chromide were found to undergo complete contraction. Santa does not report that this happened in his experiments using desoxycorticosterone. Santa also reports that the partial contraction is a lasting one, but in the present experiments many partially contracted cells were seen to expand again after 15 to 60 minutes of immersion in the desoxycorticosterone solutions.

The response of fish scale melanophores to parathyroid demonstrates that the reaction is not specific for adrenal preparations.

Our results lead us to conclude that the contraction response of fish scale melanophores does not distinguish adrenal cortical hormones from epinephrin or from the products of tissues other than the adrenal. Certainly the response is not specific for adrenal cortical hormones.

SUMMARY

The response of the melanophores of the scales of the Goldfish (*Carassius auratus*) and the Orange Chromide (*Etroplus maculatus*) to adrenal preparations and to parathyroid extract were studied. Dilutions of epinephrin chloride as high as $1:5\times10^{-5}$

elicited complete contraction of the melanophores in 1 to 5 minutes. With greater dilutions only partial contraction was observed within 45 minutes. Parathyroid extract also caused contraction. On the other hand some adrenal preparations of high potency in life maintenance caused no contraction. It is concluded that the contraction of fish scale melanophores is not a specific response to potent adrenal cortical fractions.

We wish to thank Doctor Oliver Kamm of Parke, Davis & Co. for adrenal glands and Doctor R. D. Shaner of Roche Organon Inc. for desoxycorticosterone.

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INFLUENCE OF SEX AND SEX HORMONES ON THE BREAKING STRENGTH OF BONES OF MICE[1]

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THE MORPHOLOGIC or functional changes of the tissues of the body at different ages have been the subject of frequent studies (1). The osseous tissues have been studied in several ways; the changes with advancing age on the gross and microscopic structure, the chemical composition, radio opaqueness, and reaction to injury have been observed. The present experiments were conducted in part to determine the ability of bones of mice at different ages to resist fracture and their density as determined by roentgen-ray photographs.

Although a great many factors will alter osseous tissues, few will increase the formation of bone beyond the usual limits. An increased proliferation and calcification of osseous tissue, in excess of the normal, occurred in birds and some mammals which received estrogenic hormones (2–11). The continued injection of estrogens into mice resulted in long bones in which the marrow was largely replaced by osseous tissue (2–4), the ash content was increased and the calcium : phosphorus ratio altered (12). The simultaneous injection of testosterone propionate prevented these changes in mice (13). The influence of sex hormones upon the strength and density of the bones of mice at different ages has also been investigated.

MATERIALS AND METHODS

The 'breaking strength' of both femurs of 241 mice which had received estrogenic or androgenic hormones and of 162 untreated controls was determined. The mice were either hybrids or inbred animals of the C_3H strain.[2] They were maintained in an air-conditioned building and fed Purina fox chow and water.

The estrogen-treated[3] mice received either *a*) weekly subcutaneous injections of 16.6 μg., *b*) 25 μg., or *c*) 50 μg. of estradiol benzoate, or *d*) 10 μg., *e*) 25 μg., or *f*) 50 μg. of estradiol dipropionate or *g*) 0.25 mg. of stilbestrol or *h*) pellets of estrone implanted subcutaneously. The testosterone propionate (1.0 to 2.5 mg.) was given weekly in either one or two injections. The separation of the data on the basis of the level of estrogenic treatment will not be made as all dosage levels were effective and hence above the threshold.

The injections were started in mice from 21 to 140 days of age, and continued

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[2] The mice were obtained from the colony of Dr. L. C. Strong.

[3] The estradiol benzoate and testosterone propionate were furnished by Dr. E. Schwenk and Dr. Max Gilbert of the Schering Corp., Bloomfield, N. J.; the estrone and estradiol dipropionate by Dr. E. Oppenheimer and Mr. Mautner of Ciba Pharmaceutical Products, Inc., Summit, N. J., and the stilbestrol by the E. R. Squibb Biological Laboratories, New Brunswick, N. J.

until tumors developed or the condition of the animals indicated that death was imminent. The majority of mice were 25 to 40 days old at the time the experiments were started. The femurs were removed, freed of excess flesh and measured with a caliper to determine the length and thickness at a plane parallel with the condylar axis of the distal end. The bones were then placed in an apparatus whereby weight was progressively and continuously added to a 1 cm. span of the femur (fig. 1). The weight required to fracture the span of bone was determined. Both femurs were broken and the average of the weights used for tabulating the following data. The bones were

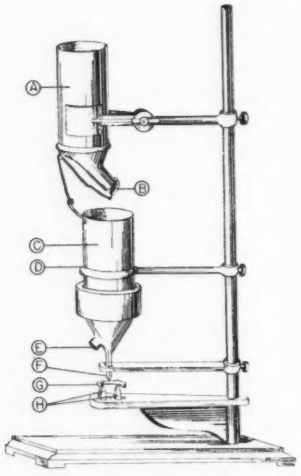

Fig. 1. Apparatus used for determination of the 'breaking strength' of the femurs of mice. (A) Container for shot; (*B*) trap door to permit passage of shot to hamper (*C*) when it is lifted above the bone (*G*) supported by the two uprights (*H*) which are set 1 cm. apart. The support (*D*) allows free movement of the weight-hamper (*C*). The shot is removed at (*E*) and weighed. The weight of the shot is added to the weight of the hamper to give the 'breaking strength' of the bones.

then either dried and photographed by roentgen-ray or they were sectioned for microscopic study. The femurs of control and experimental animals were photographed simultaneously on the same film in order to prevent errors due to differences in exposure.

OBSERVATIONS

Influence of sex upon the strength of the femurs. The bones of untreated female mice of all the hybrid groups or of the inbred strain were on the average more resistant to fracture than those of the males (fig. 2, 3; tables 1, 2). The average difference in breaking strength between the femurs of the males and females was approximately 200 gm. When the values for the males and females of the different age groups were compared, the tendency for the greater strength to be in the bones of the females was still apparent (fig. 2, 3). Insufficient observations were made on mice of the younger age groups to determine whether or not the sex differences appeared at an early age.

Effect of age on breaking strength. The breaking strength of the femurs of both male and female mice decreased in each group beyond the age of 300 days. The greatest strength of the femurs of the hybrids was apparently attained between 100 and 200 days (fig. 2), and of the C_3H mice between 300 and 400 days (fig. 3).

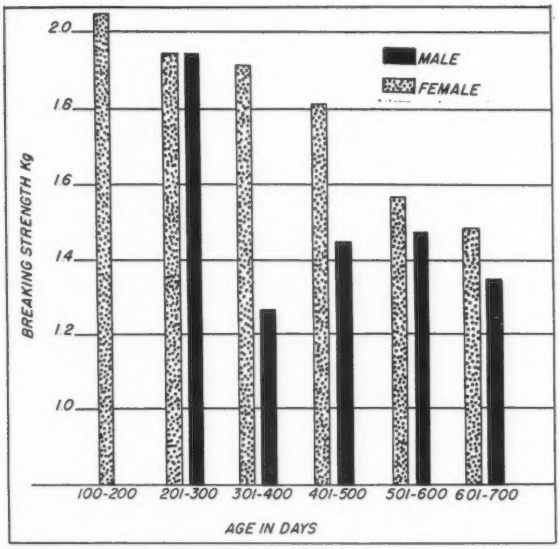

Fig. 2. AVERAGE 'BREAKING STRENGTH' OF THE FEMURS of control male and female mice of the hybrid groups at different ages. See table 2 for numbers of animals in the different groups.

Variation in breaking strength between the two femurs of the same animal. In order to determine whether the variations in breaking strength were attributable to inconsistency of technic or to real differences between the strength of the two femurs of the same animal, the mean and range of the differences between the strongest and weakest femurs were recorded. The range of the differences was quite large but the

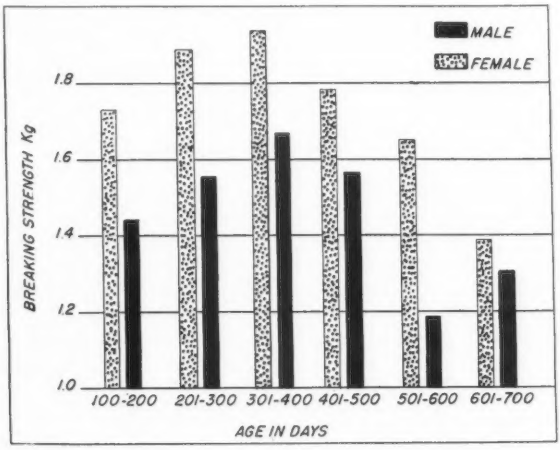

Fig. 3. AVERAGE 'BREAKING STRENGTH' OF THE FEMURS of control male and female mice of the C_3H strain at different ages. See table 2 for the numbers of animals in the different groups.

TABLE 1. MEAN BREAKING STRENGTH OF THE FEMURS OF ESTROGEN-TREATED AND CONTROL MALE AND FEMALE MICE OF SEVERAL GROUPS

Sex	Males			Females			Male and Female		
Designation of group[1]	No.	Average age, days	Average strength, kg.	No.	Average age, days	Average strength, kg.	No.	Average age, days	Average strength, kg.
Treated									
CC_1	12	504	2.80	15	565	2.22	27	538	2.48
CC_2	18	483	2.56	14	522	2.41	32	500	2.49
AC_1	7	286	2.51	6	244	2.26	13	267	2.39
AC_2	16	250	2.70	4	224	2.56	20	261	2.68
HC_2	10	231	3.01				10	231	3.01
HC_1	2	327	3.41	1	209	2.59	3	287	3.14
A_{71}	7	214	3.09	6	257	2.49	13	234	2.81
A_{72}	6	274	2.83	1	226	1.81	7	267	2.67
C_3H	42	371	2.21	29	283	2.03	71	339	2.13
C_3HTP				7	527	1.16	7	327	1.16
C_3HTPO	18	479	1.65	24	464	1.40	42	470	1.51
Control									
CC_1	8	566	1.34	8	594	1.54	16	580	1.44
CC_2	3	498	1.62	10	363	1.83	13	394	1.78
AC_1				5	275	2.08	5	275	2.80
AC_2				12	211	2.23	12	211	2.23
HC_2	1	225	1.95	11	232	1.93	12	231	1.93
A_{71}				10	236	1.73	10	236	1.73
C_3HV				44	533	1.61			
C_3H	35	448	1.40	34	337	1.91	113	467	1.65

[1] All groups except those marked C_3H were composed of hybrid mice. TP refers to mice which had received testosterone propionate. TPO refers to mice which had received both estrogen and testosterone propionate. V refers to virgin mice.

mean of the differences was small (table 3). The greatest mean difference in breaking strength for any group of control mice was 173 gm. and the mean difference for all groups was less than 150 gm. Assuming that the breaking strength of both femurs from the same mouse was equal, the average technical error did not exceed 150 gm. There is, however, some reason to doubt that the 2 femurs from the same animal

TABLE 2. AVERAGE BREAKING STRENGTH OF THE FEMURS OF MALE AND FEMALE MICE FROM SEVERAL GROUPS AT DIFFERENT AGES

Group	Sex	Treatment	100–200 Days		201–300 Days		301–400 Days		401–500 Days		501–600 Days		601–700 Days	
			No. of mice	Breaking strength	No. of mice	Breaking strength	No. of mice	Breaking strength	No. of mice	Breaking strength	No. of mice	Breaking strength	No. of mice	Breaking strength
				kg.		kg.		kg.		kg.		kg.		kg.
Hybrid	M	C			1	1.95	1	1.26	3	1.45	4	1.47	3	1.36
Hybrid	F	C	11	2.05	27	1.95	6	1.91	2	1.82	4	1.58	5	1.48
Hybrid	M&F		11	2.05	28	1.95	7	1.82	5	1.60	8	1.52	8	1.43
Hybrid	M	I	8	2.80	28	2.86	13	2.84	17	2.66	10	2.61	2	2.70
Hybrid	F	I	3	2.37	11	2.35	4	2.54	7	2.24	16	2.34	6	2.46
Hybrid	M&F	I	11	2.69	39	2.72	17	2.88	24	2.53	26	2.44	8	2.40
C_3H	M	C	5	1.45	3	1.56	5	1.67	4	1.57	7	1.19	8	1.31
C_3H	F	C	2	1.73	10	1.89	15	1.94	20	1.79	19	1.66	7	1.39
C_3H	M&F	C	3	1.57	10	1.80	20	1.86	24	1.75	26	1.53	15	1.35
C_3H	M	I	4	2.05	11	2.19	6	2.24	15	2.28	6	2.14		
C_3H	F	I	2	2.27	19	1.99	6	2.04	1	2.19	1	2.07		
C_3H	M&F	I	6	2.13	30	2.07	12	2.14	16	2.28	7	2.13		

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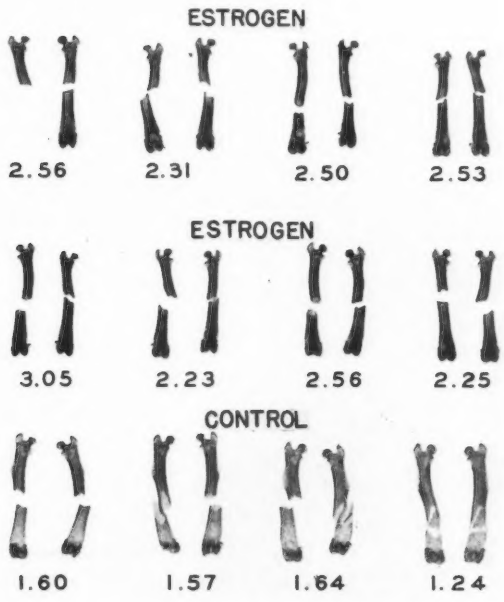

Fig. 4. Roentgen-ray photographs of femurs of estrogen-treated and control mice of the CC_2 groups and the breaking strengths in kg. All estrogen-treated mice received 16.6 μg. of estradiol benzoate weekly. Other details are given in table 4.

should break when under the same stress, as will be mentioned when the estrogen-treated animals are discussed.

Site of the fracture. This was most frequently at the mid-point of the span of the femur at the point at which the weight was applied. The bones usually broke into

TABLE 3. MEAN AND RANGE OF VARIATIONS IN BREAKING STRENGTH BETWEEN THE TWO FEMURS OF INDIVIDUAL MICE

Group of Mice	No. of Mice	Difference in Breaking Strength of the Two Femurs of Individual Mice	
		Mean (gm.)	Range (gm.)
		Injected	
CC_2	32	292	0–1160
CC_1	27	220	0–925
AC_1	13	303	0–1245
HC_1	3	230	90–500
A_{71}	13	211	0–525
A_{72}	7	299	0–970
HC_2	10	144	0–500
AC_2	20	208	0–670
		Controls	
CC_2	13	138	20–275
CC_1	16	141	0–555
AC_1	5	142	35–205
A_{71}	10	173	0–455
AC_2	12	131	0–400
HC_2	12	143	0–410

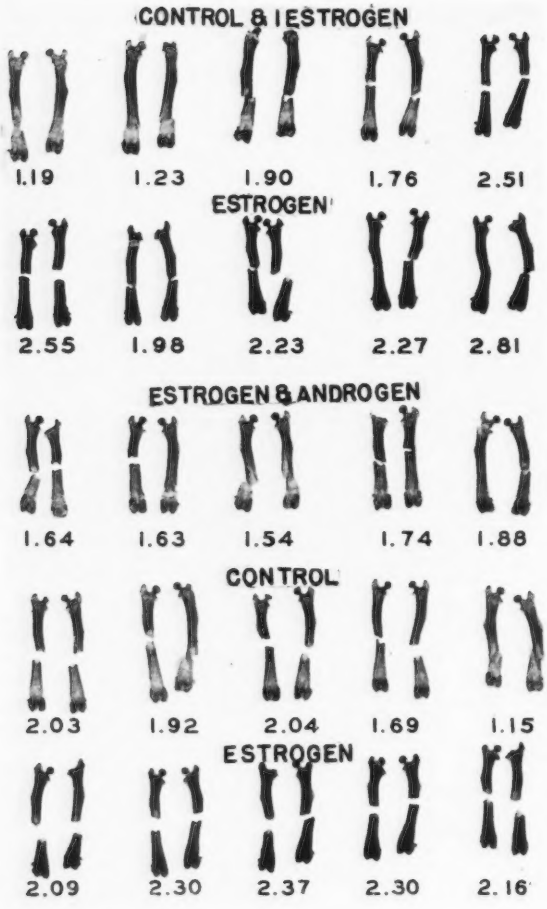

Fig. 5. Roentgen-ray photographs of femurs of estrogen-treated, estrogen and androgen-treated and control C_3H mice and the breaking strength in kg. The estrogen-treated mice received from 10 to 50 μg. of estradiol benzoate weekly in one injection. The estrogen and androgen-treated mice received 1.25 mg. of testosterone propionate and 16.6 μg. of estradiol benzoate weekly. Other details are given in table 4.

two pieces without shattering, the fracture being somewhat oblique. The weakest bones occasionally broke obliquely and between the center of the span and the distal end of the femur. These weaker bones sometimes shattered and were apparently crushed rather than broken.

The roentgen-ray photographs of the femurs of the control mice did not reveal differences in opaqueness of the bones which could be associated consistently with the values for the breaking strength. The differences between the strongest and the weakest bones were usually apparent in the films (fig. 4, 5).

Influence of estrogen on the breaking strength of femurs. The femurs of estrogen-treated mice of all groups were stronger than those of the untreated controls (tables 1, 2; fig. 6, 7, 8). The femurs of the males which had received estrogen were almost

always stronger than those of the treated females. The difference between the breaking strength of the femurs of the control and estrogen-treated animals tended to increase with advancing age. In other words, the femurs of these animals did not decrease in their capacity to resist fracture at the same rate or to the same extent as the controls (fig. 6, 7, 8).

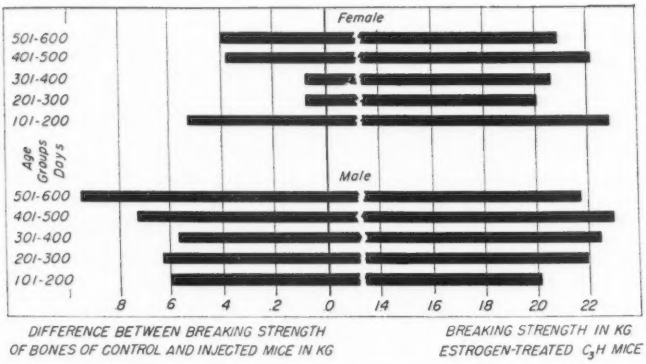

Fig. 6. AVERAGE 'BREAKING STRENGTH' OF THE FEMURS of estrogen-treated male and female C3H mice at different ages (at the right) and the differences between the 'strength' of the bones of the controls and estrogen-treated animals of the same age groups (at the left).

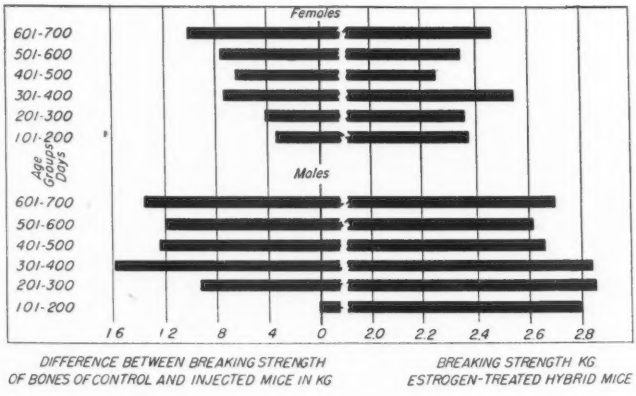

Fig. 7. AVERAGE 'BREAKING STRENGTH' OF THE FEMURS of estrogen-treated male and female mice of the hybrid groups (at the right) and the differences between the 'strength' of the bones of the controls and estrogen-treated animals of the same age groups (at the left).

The femurs of the estrogen-treated females showed a less striking relative and a less striking absolute increase in breaking strength than did those of the males of similar groups. The breaking strength of the femurs was relatively greater in the injected animals of the older age groups.

The range of variations between the breaking strength of the two femurs of individual mice was much greater than in the control series (table 3). The mean variation was always quite small. The extreme differences in breaking strength were

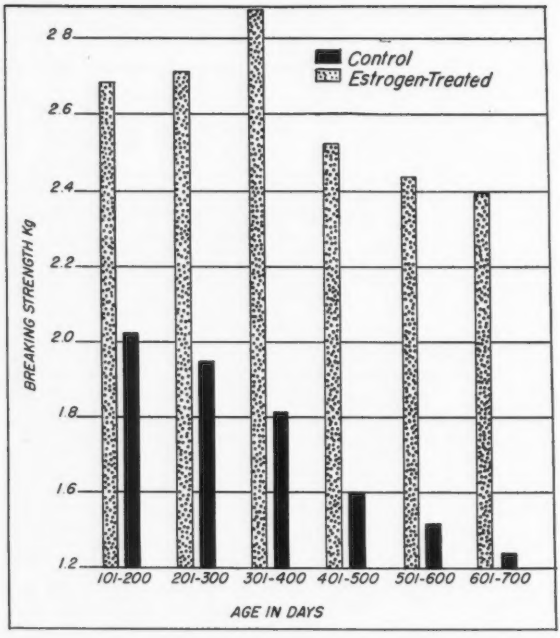

Fig. 8. AVERAGE 'BREAKING STRENGTH' OF THE FEMURS of the hybrid male and female mice of the estrogen-treated and control groups at different ages.

limited to very few mice. Only 5 of the 135 hybrid mice showed variations of over 900 gm. In each of these 5 mice one femur had a localized decalcified area near the distal end and fractures occurred through these areas. The differences in breaking strength between the femurs of these mice could be attributed to local alteration of the bony tissue and not to a generalized osteoporosis.

TABLE 4. BREAKING STRENGTH AND MEASUREMENTS OF THE FEMURS OF THE 8 ESTROGEN-TREATED AND 4 CONTROL CC_2 MICE SHOWN IN FIGURE 4

No. of Photograph (left to right)	Sex	Average Breaking Strength	Breaking Strength of Femur		Age Started	Age at Death	Measurements of Femur	
			Right	Left			Right	Left
		kg.	*kg.*	*kg.*	*days*	*days*	*mm.*	*mm.*
1	M	2.56	2.34	2.77	29	444	1.56×16.2	1.56×16.2
2	F	2.31	2.31	2.31	29	481	1.35×15.5	1.34×15.7
3	F	2.50	2.54	2.47	27	508	1.44×15.9	1.46×15.9
4	F	2.53	2.72	2.34	27	544	1.46×15.8	1.48×16.0
5	M	3.05	2.97	3.17	31	322	1.46×15.4	1.49×15.6
6	F	2.23	2.27	2.20	34	426	1.45×15.5	1.47×15.4
7	M	2.56	2.50	2.62	34	445	1.47×15.6	1.47×15.6
8	M	2.25	2.16	2.34	56	469	1.45×15.5	1.40×15.5
9C	F	1.60	1.55	1.66		397	1.50×16.8	1.51×16.8
10C	F	1.57	1.40	1.75		394	1.46×17.1	1.47×17.1
11C	M	1.64	1.70	1.57		452	1.51×17.1	1.45×17.1
12C	M	1.24	1.38	1.11		498	1.54×17.4	1.53×17.4

The femurs of all estrogen-treated mice were much more radio-opaque than those of the controls. The most heavily calcified bones were usually the stronger. The amount of variation in density was always greater in the estrogen-treated than in the control groups (fig. 4, 5).

Effect of testosterone propionate upon the breaking strength of the femurs when administered alone or with estrogen. The simultaneous administration of testosterone propionate in amounts of 1.0 to 2.5 mg. weekly and estradiol benzoate (16.6 to 50 μg. weekly) largely prevented the increased strength of the bones which would have been expected had estrogen been given alone. In fact, the average values for the breaking strength were lower than those of untreated controls. The femurs of mice given testosterone propionate alone were less resistant to fracture than those of untreated mice of the same age groups. The femurs of the mice of this group were the least resistant to fracture.

TABLE 5. BREAKING STRENGTH AND MEASUREMENTS OF THE FEMURS OF CONTROL, ESTROGEN-TREATED AND ESTROGEN-ANDROGEN-TREATED GROUP OF MICE OF THE C_3H STRAIN SHOWN IN FIGURE 5

No. of Photograph (left to right)	Sex	Average Breaking Strength	Breaking Strengths		Age Started	Age at Death	Measurements of Femur	
			Right	Left			Right	Left
		kg.	*kg.*	*kg.*	*days*	*days*	*mm.*	*mm.*
1C	M	1.19	1.31	1.07		534	1.23×14.5	1.23×14.5
2C	M	1.23	1.17	1.28		587	1.36×15.6	1.36×15.6
3C	F	1.90	1.83	1.97		728	1.51×16.5	1.51×16.7
4C	M	1.76	1.54	1.99		374	1.28×15.5	1.28×15.5
5	F	2.51	2.66	2.36	33	287	1.28×13.8	1.29×13.7
6	M	2.55	2.56	2.34	34	400	1.34×14.7	1.36×14.7
7	M	1.98	2.11	1.86	41	457	—	—
8	M	2.23	2.23	2.22	50	429	1.33×14.8	—
9	M	2.27	2.24	2.30	49	402	1.32×14.6	1.32×14.6
10	M	2.81	2.80	2.82	39	368	—	—
11 TPO	F	1.64	1.68	1.62	47	500	1.23×14.1	1.23×14.3
12 TPO	F	1.63	1.48	1.78	35	499	1.27×13.7	1.28×13.7
13 TPO	F	1.54	1.52	1.56	35	471	1.25×13.8	1.26×13.8
14 TPO	M	1.74	1.98	1.52	52	422	1.35×14.8	—
15 TPO	M	1.88	1.77	1.98	35	432	1.38×14.7	1.37×14.7
16 C	M	2.03	2.12	1.93		304	1.37×16.5	1.34×16.5
17 C	F	1.92	1.89	2.06		336	1.41×16	1.35×16.3
18 C	F	2.04	1.85	2.23		296	1.30×15.2	1.32×15.2
19 C	F	1.69	1.60	1.79		303	1.30×16.3	1.29×1.63
20 C	M	1.15	1.17	1.13		609	1.33×15.3	1.31×15.4
21	M	2.09	1.88	2.30	59	510	1.31×15.2	1.33×15.2
22	M	2.30	2.78	2.32	64	416	1.35×14.9	1.33×14.9
23	M	2.37	2.40	2.33	53	432	1.26×14.4	1.23×14.4
24	M	2.30	2.61	1.99	50	424	1.42×14.6	1.3 ×14.5
25	M	2.16	1.99	2.34	34	474	1.32×14.8	1.3 ×14.5

The roentgen-ray photographs of the mice of this group showed the least degree of radio-opacity of all groups. The fractures were usually quite oblique or the bones were somewhat shattered. The diaphyseal walls were very thin.

DISCUSSION

The sex hormones were first associated with levels of serum calcium in birds (15) and reptiles (16), and more recently with the osseous tissues of birds (3, 17, 18, 19) and mice (2, 11). Estrogen increased the levels of calcium in the blood of toads, chickens and pigeons, and in the birds and mice it induced a proliferation of bone in

the medullary areas. Earlier studies had revealed that the levels of calcium in blood serum (15) and the amount of trabecular endosteal bone increased when large follicles developed in the ovaries in pigeons (17). Until recently the known effects of estrogens upon the skeletal tissues of mammals were limited to the pelves of animals of certain species. The pubes of estrogen-treated pocket gophers (20) and mice (21) became widely separated, the symphyses were replaced by interpubic ligaments. The same changes were associated with the onset of sexual maturity or the latter part of pregnancy in these two species, respectively. Thus in some birds and some mammalian species the skeletal tissues show either generalized or local alterations which could be associated with reproductive phenomena.

The influence of sex hormones on the extra-pelvic osseous tissues has been studied more recently in mice (2, 4, 9, 11) and rats (3, 22, 23). The medullae of the bones of estrogen-treated mice may be filled with compact bone (4). These changes are attained by an increased proliferation of the peritrabecular osteoblastic tissue, sclerosis of the epiphyseal cartilages and also by a proliferation of the diaphyseal endosteum (4, 9).

Two different and somewhat opposite effects of estrogenic hormones upon skeletal tissues have been mentioned, namely, removal of osseous material from some areas such as parts of the pelvis and the increased formation of bone in other areas. These changes might be considered to provide for the enlargement of the birth canal and to provide a reserve of calcium salts for the drain of egg-shell formation in birds, or fetal calcification and lactation in mammals. These opposite responses in the pelvic and other skeletal tissues also show that one and the same inciting agent may result in different reactions at the same time, depending upon other factors which must localize and determine the nature of the response. The variation in response of the skeletal tissues of mice to estrogens was unique when contrasted to the responses of other tissues. Although estrogens may incite local stimulation of epithelial or connective tissues, such as the uterus and mammary glands, it is not known to prevent the stimulation or facilitate the destruction of other connective tissues in the same body.

The response of animals or their tissues to administered hormones does not necessarily imply that these substances are effective when intrinsically produced, or that intrinsically produced hormones could attain adequate levels to affect such responses. The resorption of the pubes and formation of an interpubic ligament occurs during pregnancy and after injections of estrogen. The greater average strength of the femurs of female mice permit the interpretation that the intrinsic humoral environment may be responsible for these conditions. The same tendency is also indicated by the greater ash and bone salt content of female rats (24, 25) and mice (12) than of the males.

The bones of mice decrease in strength with advancing age. These changes with advancing age might result from a decrease in the levels of intrinsic hormone or independent of any change in estrogen level. The former possibility was indicated because injected estrogens largely prevented the decrease in breaking strength with advancing age.

If the assumption is made that estrogens are associated with the strength of bones of untreated mice as stated above, then how is this change brought about? Estrogens may directly stimulate the proliferation of osteogenic tissues or produce sclerosis of epiphyseal cartilage. This seems, at this time, to be the most logical explanation for the osseous changes, although the localization of the changes to the endosteum and peritrabecular osteoblasts and not the periosteum indicates that this is not the whole answer. Estrogens may stimulate the undifferentiated elements of the medullary tissues to become osteoblasts.

Bones atrophy following paralysis or immobilization of parts (26). General activity and muscular tonicity may decrease in mice with advancing age and this could account, possibly, for the general thinning of the walls of the bones (27). Estrus and injected estrogens, in short experiments, cause a greatly increased activity of rats (28). It is not known whether a similar hyperactivity would occur in mice and whether it would persist in prolonged experiments.

Estrogens elicit hyperemia of certain tissues of the body of some animals. It may be that the vascular supply of the myeloid and endosteal tissues is altered, favoring osteoblastic proliferation and calcification.

The injection of estrogens in large amounts resulted in stunting of the body growth and a precocious sclerosis of the epiphyseal cartilage (3, 9). The bones of the estrogen-treated mice were smaller than those of the controls when the injections were started in the younger animals. The stunting effect of the estrogen was not apparent when older mice were used. The average length and diameter of the bones of the estrogen-treated mice were smaller than in those of the controls, however. The proliferation at the epiphyseal cartilage and the addition of periosteal bone was, therefore, inhibited to some extent. The addition of androgen was not antagonistic to the inhibition of the epiphyseal or periosteal proliferation observed in estrogen-treated mice, but rather augmented it. The bones of many estrogen-treated mice increased somewhat in length after the experiments were started but 'clubbing' was never observed. The subperiosteal osseous tissue near the epiphysis was systemically removed to maintain the normal shape of the bone.

All of the mice were fed a commercial diet adequate in ash. Rats fed low salt diets had femurs only one-third to one-half as strong as those of controls on an adequate salt intake (29). The restriction in calories of a diet adequate in salt did not affect the breaking strength appreciably. Since both the control and estrogen-treated mice of both sexes and of all ages were fed *ad libitum* nothing is known of their food intake. It does not seem probable that dietary factors could be responsible for the sex differences or the influence of the hormones on the bones. The larger amount of calcium and phosphorus consumed may be retained in the estrogen-treated mice as indicated in clinical experiments (30).

SUMMARY AND CONCLUSIONS

The 'breaking strength' of the femurs of 241 mice which had received estrogens (estradiol benzoate, estradiol dipropionate, stilbestrol or estrone) or estradiol benzoate plus testosterone propionate was determined under uniform conditions. The breaking strength of the femurs of 162 untreated mice of the same stocks was also determined.

The femurs of untreated mice declined in strength as the mice advanced in age. The bones of the females were stronger than those of the males. The most radiopaque femurs in general showed the greatest resistance to fracture.

The breaking strength of the femurs was much greater in the estrogen-treated mice than in the controls of the same age groups. Estrogen-treated male mice had stronger femurs than females similarly treated. The bones of the old estrogen-treated mice were relatively stronger than those of the younger estrogen-treated mice when compared to controls of the corresponding age groups.

The simultaneous injection of testosterone propionate and estrogen prevented the increased strength observed in mice given estrogens alone.

The bones of female mice given testosterone propionate were much weaker than the bones of untreated controls of the same age groups.

The presence of the ovaries and testes or the injected estrogenic and androgenic hormones influenced the strength of the bones of mice.

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EFFECT OF COMBINING ESTROGEN WITH LETHAL DOSES OF ROENTGEN-RAY IN SWISS MICE[1]

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SEVERAL years ago in studies on the experimental induction of leukemia (1, 2) in mice by roentgen-rays and estrogen, it was observed that estrogen-treated mice were able to withstand larger doses of radiation than mice which had not received estrogens prior to irradiation. Experiments were conducted in which both roentgen-rays and estrogen were supplied to the same mice. A much higher percentage of the mice which had received estradiol benzoate prior to irradiation, tolerated the treatment (table 1). This gave rise to considerations regarding the possible protective effect of estrogens in radiation reactions. The present study was undertaken in an effort to provide an answer to this question.

TABLE 1. SURVIVAL OF MICE OF SEVERAL INBRED STRAINS SUBJECTED TO IRRADIATION WITH 400 ROENTGEN UNITS WITHOUT, OR FOLLOWING ESTROGEN

Treatment	No. of Mice	No. Surviving Irradiation 40 Days or More	Percentage Surviving Irradiation 40 Days or More
Roentgen rays alone	68	10	14.7
Estrogen 9–11 days before irradiation	54	31	57.4

METHODS

In the earlier experiments, mice of several different inbred strains were used. All mice were 40 to 60 days of age and litter mates were used in each group. The mice were irradiated in low cardboard boxes, each receiving 400 roentgen units whole body radiation. The radiation was delivered by a 200 K.V. roentgen-ray tube at a target distance of 50 cm. The output was 68 roentgen units per minute and the rays were filtered through 1 mm. of aluminum and 0.5 mm. of copper. Certain of the mice received one injection each, of 16.6 μg. of estradiol benzoate[2] 9 to 11 days prior to irradiation.

In the more recent experiment 204 healthy adult male Swiss mice were used. This strain was selected because of the natural immunity of such mice to typhoid, and because of their unusual vigor. The weight of the mice did not vary more than 5 gm. and the average was 25 gm. each. They were divided into 3 groups of 68 mice each.

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[2] The estradiol benzoate (Progynon B) was supplied through the courtesy of Doctor E. Schwenk by the Schering Corp., Bloomfield, N. J.

The source of the roentgen-rays was a General Electric 220 K.V. Maximar roentgen-ray tube. With a target-skin distance 50 cm. the output for some of the runs was 39 roentgen units per minute, and for others 60 roentgen units per minute. The rays were filtered through 0.5 mm. of copper and 1 mm. of aluminum. The Victoreen roentgenometer was used for all dose measurements.

Whole body irradiation of the mice was accomplished by packing them into a box in such a way that the mice lay side by side in a single layer. The target-skin distance was measured and the dosage calibrated to the inside center of the box. *Group I*, the controls, was given 500 roentgen units whole body radiation in the manner described. *Group II* was given 550 roentgen units whole body radiation which was followed immediately by injection into the leg muscle of 0.05 cc. of estradiol

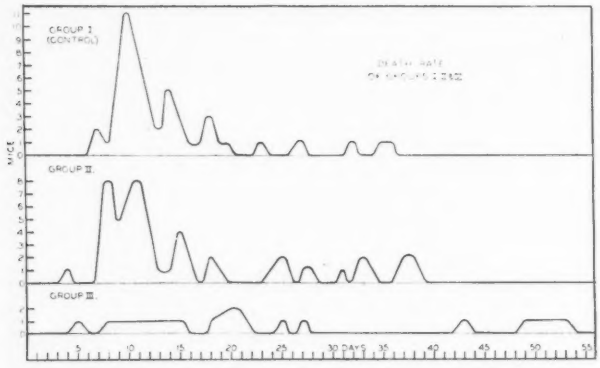

Fig. 1. DEATH RATE OF MICE receiving roentgen ray irradiation. Group I. Controls. Group II. Irradiation was followed by treatment with estradiol benzoate intramuscularly. Group III. Estradiol benzoate intramuscularly 9 days prior to irradiation.

benzoate, containing 10,000 R.U. (1.66 mg.) per cc. *Group III* was given 0.05 cc. (0.083 mg.) of estrogen by injection into the leg muscle, and 9 days later was given 550 roentgen units whole body radiation.

The mice of all groups were observed for a period of 60 days following irradiation, and autopsies were performed on those which died during the experimental period.

RESULTS

Results of the earlier experiments are summarized in table 1. Results of the present work are shown graphically in figure 1 and are summarized in table 2. Table 3 shows the rate of death for each of the 3 groups used in the present study. In all cases autopsies revealed similar gross conditions, namely: dilation of the small intestine from pylorus to cecum, with many injected blood vessels; hemorrhages into subcutaneous tissues; hemorrhagic injection of lymph nodes; atrophy and spotting (due to focal necrosis) of liver; atrophy of the spleen. Congestion of the lungs was not infrequently present. Death was always preceded by ruffling of the fur and arching of the back, and was frequently preceded by diarrhea. The foregoing pathological changes may be considered typical of radiation damage in mice (4).

The controls, *Group I*, showed a rate of survival of 20.6 per cent. This group shows the sharpest peak with regard to deaths, 33.3 per cent having been lost on the tenth and eleventh days. The average length of survival for the group was 23.9 days.

Group II had a rate of survival less than one-half that of the controls, namely, 11.7 per cent. This group shows the highest death rate within the shortest time after irradiation, 50 per cent having been lost between the eighth and twelfth days. The average length of survival for the group was 20.4 days.

Group III showed a rate of survival more than 3 times as high as in the controls, or 75.0 per cent. The curve expressing rate of death shows no peak, and while the initial death in the group occurred at approximatly the same interval after irradiation as in the other two groups (4 days), it was not followed by a rise. On the contrary, the total deaths are distributed throughout the experimental period, while in

TABLE 2. RATE OF SURVIVAL OF ADULT MALE SWISS MICE FOLLOWING IRRADIATION WITH 550 ROENTGEN UNITS UNDER DIFFERENT CONDITIONS

Treatment	No. of Mice	No. Surviving Irradiation 40 Days or More	Percentage Surviving Irradiation 40 Days or More
Roentgen-rays alone	68	14	20.6
Estrogen and roentgen-rays simultaneously	68	8	11.7
Estrogen 9 days before irradiation	68	51	75.0

TABLE 3. DEATH RATE OF SWISS MICE OF THE THREE EXPERIMENTAL GROUPS BY 5-DAY PERIODS

DAYS	GROUP I CONTROLS	GROUP 2	GROUP 3
1– 5	0	1	1
6–10	17	21	5
11–15	23	20	4
16–20	7	5	3
21–25	1	3	3
26–30	1	2	1
31–35	2	4	0
36–40	1	4	0
41–45	0	0	1
46–50	0	0	2
51–55	0	0	3
56–60	0	0	0

Groups I and *II* no deaths occurred later than the thirty-ninth day following irradiation. The average length of survival for the group was 47.8 days, or more than twice the survival period of the controls and of *Group II*.

DISCUSSION

The dose of radiation alone was sufficient to cause death within 39 days in 79.4 per cent of the mice in the present experiments which were not given estrogen. The administration of 0.05 cc. (0.083 mg. of estradiol benzoate) of estrogen simultaneously with the same dose of roentgen-ray, enhanced the radiation effect by causing death *a*) in a larger number of mice and *b*) within a shorter time after irradiation. On the other hand, when the same dose of estrogen was administered 9 days prior to a lethal dose of roentgen-ray, death occurred in a far smaller number of mice than in the control group, and the average period of survival was lengthened markedly. These facts suggest the possibility that the administration of estrogen prior to irradiation with roentgen-ray, exercises a protective effect against the destructive action of massive doses of roentgen-ray.

Roentgen-rays apparently damage the lymphoid, myeloid and gastro-intestinal tissues most extensively when administered in minimal lethal or sublethal doses (4). The partial destruction of these tissues probably is responsible directly or indirectly for the death of the animals treated with the amounts of radiation used in the present experiments. How estrogens might alter these toxic effects of roentgen-rays is unknown. The administration of large amounts of estrogens to dogs for prolonged periods resulted in a profound myeloid aplasia (5). A degeneration of the thymus and probably other lymphoid tissues occurred in rats injected with estrogen (6). Also, because simultaneous roentgen-irradiation and injection of estrogen did not prolong life but actually increased the toxicity of the treatment—it can not be assumed that the effect of roentgen-rays and estrogen are antagonistic. The possibility exists that after an acute toxic reaction of the lymphoid tissues to estrogens a compensating reaction occurs and is prevalent at the time when the delayed irradiation is imposed; or that the tissue persisting after estrogenic treatment is less susceptible to the destructive action of roentgen-rays.

SUMMARY

Five hundred and fifty roentgen units of whole body radiation to Swiss mice produces a lethal effect within 40 days on 79.4 per cent of a group so treated. Such a dose of radiation accompanied immediately by intramuscular injection of 0.05 cc. of estrogen (10,000 rat units per cc.) has a lethal effect on 88.2 per cent of similar mice. Five hundredths of a cc. of estrogen injected intramuscularly in such mice 9 days prior to 550 roentgen units whole body radiation, results in death to 25 per cent of the group within 40 days, and to 33.7 per cent within a period of 60 days after irradiation.

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A COMPARATIVE ASSAY OF DESOXYCORTICOSTERONE ACETATE AND ACETOXYPREGNENOLONE IN THE ADRENALECTOMIZED DOG

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THE ACTIVITY of desoxycorticosterone acetate in maintaining the life of adrenalectomized animals has been abundantly demonstrated (1–4). Acetoxypregnenolone (pregnene (5:6) diol (3, 21) one (20) 21-mono-acetate), its immediate precursor in laboratory synthesis (5), has been reported *a*) to maintain the life of adrenalectomized dogs (6), *b*) to increase the work performance of adrenalectomized rats (7), and *c*) to prolong the life of immature adrenalectomized rats (8). The investigations of Selye (8) on young rats indicated that acetoxypregnenolone may be one-third to one-half as active as desoxycorticosterone acetate (9). The test of acetoxypregnenolone for life-prolonging activity in dogs, conducted by Waterman et al. (6), did not show as close an approximation in strength of acetoxypregnenolone to desoxycorticosterone acetate. The test is open to criticism, however, because the period of 4 days for one dosage level is too short to gain a reliable estimate of the activity of the preparation, and because the salt content of the diet, which is now known to affect the hormone requirement greatly (4), was not given. In order to determine more accurately the activity of acetoxypregnenolone as compared to desoxycorticosterone acetate, an assay of these substances has been conducted in a fashion designed to overcome these objections.

TECHNIC

Three adult male dogs adrenalectomized in two stages were used. Details as to the diet and care have been described previously (4). The diet consisted chiefly of meat and contained approximately 1 per cent NaCl. The assay method was essentially that of Harrop, Pfiffner, Weinstein and Swingle (10) as modified in this laboratory (4). Blood was obtained from an anterior leg vein for determinations of the non-protein nitrogen and packed cell volume at intervals at 2 to 7 days, at least 16 hours after food and hormone injection which was given subcutaneously. Each injection period was 13 to 15 days, the dose being reduced in successive periods until signs of insufficiency appeared. The desoxycorticosterone acetate[1] was assayed before and, in two of the dogs, after administration of acetoxypregnenolone. The assay was started one week after the removal of the second adrenal. The daily dose of desoxycorticosterone acetate used in the first period was 1.66 mg. in 0.33 cc. of sesame oil. The dosage was reduced at the start of the second period by substituting a preparation containing 1 mg. in 1 cc. of oil, in preference to reducing the amount of the more concentrated preparation used during the first period. This was done in order to

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[1] Both the desoxycorticosterone acetate and acetoxypregnenolone used in this investigation were supplied by the Schering Corp., Bloomfield, N. J., through the kindness of Dr. E. Schwenk.

be sure that the volume of the injected vehicle containing desoxycorticosterone acetate would approximate the volume of acetoxypregnenolone solution to be injected. The resting heart rate was also recorded, since it had been found that tachycardia often develops early with an inadequate dosage of hormone and it was desirable to ascertain how closely this followed other indications of inadequate treatment.

RESULTS

The results of the assays of both desoxycorticosterone acetate and acetoxypregnenolone are summarized in table 1 and illustrated in figures 1 and 2. In *dogs* 1 and 3 the

TABLE 1. COMPARATIVE ASSAY OF DESOXYCORTICOSTERONE ACETATE AND ACETOXYPREGNENOLONE

Dog No.	Weight	Assay Period	Desoxycorticosterone Acetate			Acetoxypregnenolone		
			Solution		Result	Solution		Result
	kg.	(13–15 dy.)	cc.	mg.		cc.	mg.	
1	9.5	First	0.33	1.66	Adequate			
		Second	1.00	1.00	Inadequate			
		Third				2	10	Adequate
		Fourth				1	5	Inadequate
		Fifth	1.50	1.50	Adequate			
2	11.5	First	0.33	1.66	Adequate			
		Second	1.00	1.00	Questionable			
		Third	0.50	0.50	Inadequate			
		Fourth				1	5	Inadequate
		Fifth				0.5	2.5	Inadequate
3	10.5	First	0.33	1.66	Adequate			
		Second	1.00	1.00	Inadequate			
		Third				2	10	Inadequate
		Fourth				1	5	Inadequate
		Fifth	1.50	1.50	Adequate			

Summary: Ratio of adequate dose of desoxycorticosterone acetate to adequate dose of acetoxypregnenolone: *Dog* 1, 1:6.6; *Dog* 2, 1:5.0; *Dog* 3, 1:6.6.

assays were reasonably clear cut, 1.5 mg. of desoxycorticosterone acetate in 1.5 cc. of sesame oil being an effective maintenance dose as judged by maintenance of weight, prevention of an increase of non-protein nitrogen of more than 50 per cent of the normal (35 mg. per cent), maintenance of the level of packed cell volume and of heart rate. *Dog* 2 had a lower requirement for desoxycorticosterone acetate and showed less clear cut changes on an inadequate dosage although withdrawal of hormone therapy, and subsequent autopsy, demonstrated the absence of cortical tissue as in the other two dogs.

Acetoxypregnenolone in a 10-mg. dose daily effectively maintained *dog* 1 for a 2-week period. Evidence of its activity was seen in the other 2 animals given acetoxypregnenolone, as judged by the failure of signs of severe adrenal insufficiency to develop during the first 2-week period of acetoxypregnenolone treatment. Definite evidence of inadequacy of the therapy was apparent during this period in *dog* 3, receiving 10 mg. of acetoxypregnenolone daily, and in *dog* 2 receiving 5 mg. daily. Signs of severe adrenal insufficiency developed in both of these dogs during a subsequent 10-day period when the dose had been halved.

The packed cell volume followed other changes indicative of the state of the animal in a general way, but occasional values were obtained which were thought not to represent resting values. In all 3 dogs there was a marked general decline in the packed cell volume over the assay period of 3 months.

An increase in the resting heart rate generally accompanied other signs of inadequate therapy but this was not as regular or as early an indication as an increase in non-protein nitrogen or packed cell volume, failing to increase in some instances despite definite hemoconcentration. The development of more severe adrenal insufficiency always led to some degree of tachycardia. Treatment was instituted before the bradycardia (11) of adrenal insufficiency or other sign of collapse occurred. The heart rate did not at once return to a normal resting rate with the relief of hemoconcentra-

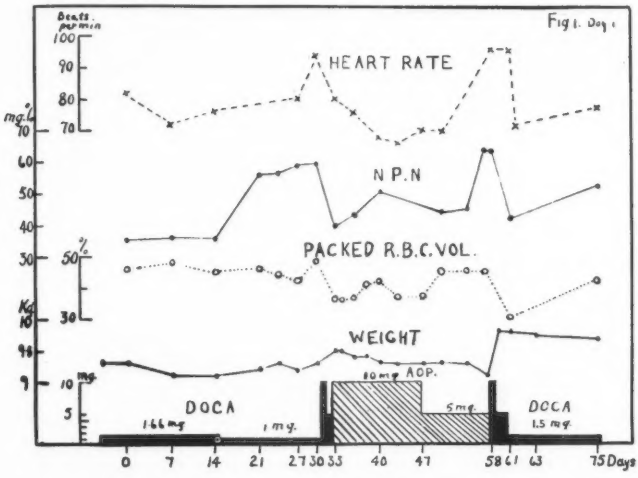

Fig. 1. Findings in dog 1: Dec. 30, 1941, second adrenal removed. Cortin and desoxycorticosterone acetate daily until Jan. 6, 1942, when assay using 1% NaCl diet and daily subcutaneous injections of desoxycorticosterone acetate, 1.66 mg. in 0.3 cc. of sesame oil, started. On the 15th day dosage reduced to 1 mg. in 1 cc. of sesame oil daily. On the 30th day signs indicate inadequate dosage. Thirty-first day 6 gm. of NaCl extra given on food and 10 mg. of desoxycorticosterone acetate. Thirty-second day, no extra NaCl; 5 mg. of desoxycorticosterone acetate. Assay of acetoxypregnenolone begun of 33rd day: 10 mg. in 2 cc. of sesame oil injected subcutaneously daily. On 47th day dosage reduced to 5 mg. in 1 cc. of sesame oil daily. On 57th day signs indicate inadequate dosage. Six gm. of NaCl extra given on food and 10 mg. of desoxycorticosterone acetate on *day* 58. Five gm. of sodium chloride extra and 5 mg. of desoxycorticosterone acetate, *day* 59. *Day* 60, no extra NaCl; 5 mg. of desoxycorticosterone acetate. On 61st day 1.5 mg. of desoxycorticosterone acetate in 1 cc. of sesame oil begun. Assay terminated on 75th day.

tion but often showed a lag of a day or two before subsiding to the normal slow rate. This is particularly noticeable in figure 1 at the sixtieth day.

DISCUSSION

If acetoxypregnenolone were as closely comparable in activity to desoxycorticosterone acetate as Selye's findings suggest, it might serve as a cheaper source of effective hormone therapy for Addison's disease. It might also be considered as having an advantage over desoxycorticosterone acetate in that, as Selye (8) showed, it does not cause adrenal atrophy. This can be considered only a hypothetical asset since there is no substantial evidence that desoxycorticosterone acetate causes atrophy of the cortex in dogs or man in therapeutic dosage. The findings reported here indicate that acetoxypregnenolone is less than one-sixth as active as desoxycorticosterone acetate; therefore, it cannot be considered as a profitable substitute.

The maintenance dose for desoxycorticosterone acetate in the dogs in the present study—1.5, 1.5 and 1 to 1.5 mg. daily, subcutaneously—is similar to that found in 4 dogs previously studied (4) in which the average requirement was 1.55 mg. daily. In that study the material was given intramuscularly in a smaller volume of oil in a concentration of 5 mg. to 1 cc.; a concentration of 1 mg. in 1 cc. was used in the critical phases of the present assay. In the 4 animals receiving the desoxycorticosterone acetate intramuscularly in the concentrated form and, therefore, in a smaller volume of oil, the non-protein nitrogen and other changes indicative of inadequate treatment

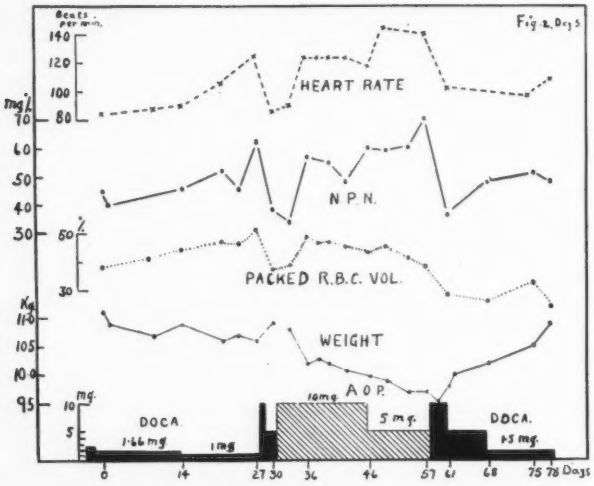

Fig. 2. Findings in *dog* 3: Dec. 30, 1941 second adrenal removed. Cortical extract and desoxycorticosterone acetate until Jan. 3, 1942 when adrenal cortical extract stopped. Jan. 6, assay begun: 1 per cent NaCl diet and daily subcutaneous injections of desoxycorticosterone acetate, 1.66 mg. in 0.33 cc. of sesame oil started. On 14th day dose changed to 1 mg. in 1 cc. of sesame oil daily. Signs of inadequate dosage on *day* 27. *Day* 28, given 6 gm. of NaCl extra on food and 10 mg. of desoxycorticosterone acetate. *Day* 29 and 30, 3 gm. of NaCl extra and 5 mg. of desoxycorticosterone acetate daily. On 31st day assay of acetoxypregnenolone begun: 10 mg. in 2 cc. of sesame oil injected subcutaneously daily. On the 46th day signs indicate inadequate dosage but, nevertheless, dosage of acetoxypregnenolone reduced to 5 mg. in 1 cc. of sesame oil daily. On 57th day, signs of inadequate dosage more marked, except packed red cell volume which is less. *Days* 58, 59 and 60 extra NaCl and 10 mg. of desoxycorticosterone acetate daily. *Days* 61 to 67 5 mg. of desoxycorticosterone acetate daily. On the 68th day reduced to 1.5 mg. in 1.5 cc. of sesame oil. Assay terminated on 78th day. Diet constant in amount and all of it was always eaten.

were sharper, probably because the smaller volume of the injected vehicle insures more rapid absorption and therefore less continuous action of the hormone contained.

The packed cell volume, as already indicated, increases with the non-protein nitrogen. A liability to error lies in the tendency of this value to rise with exercise and excitement in the dog. It is dangerously easy, therefore, to obtain results which do not represent the value in the resting state. Even with dogs accustomed to being handled, this factor enters, as we have observed when taking repeated blood samples at half-hour intervals for blood volume studies, considerably lower values usually being obtained from the second, third and fourth samples. This minimizes the value of observations depending on packed cell volume estimations made on a single sample.

As in our previous study (4), a progressive decline in packed cell volume was noted in the animals in the present study. This can only be attributed to the develop-

ment of an anemia, since it is observed during periods of treatment when over-dilution cannot be considered a factor. Pfiffner et al. (12) have previously noted the development of anemia in adrenalectomized dogs. It has been our impression that it is more often apparent in dogs maintained on desoxycorticosterone acetate than in those receiving whole gland extract. This may be in part because we have become impressed with the phenomenon recently and, therefore, are on the lookout for it.

The explanation for the failure of the heart rate to follow hemoconcentration more closely in some animals than others is not apparent.

CONCLUSIONS

Assay of desoxycorticosterone acetate and acetoxypregnenolone in 3 adrenalectomized dogs receiving a meat diet containing approximately 1 per cent sodium chloride shows that acetoxypregnenolone has less than one-sixth the activity of desoxycorticosterone acetate. The value of the packed cell volume in deciding the adequacy of the substitution therapy, the general decline in the proportion of red blood cells in the whole blood and the response of the heart rate to hemoconcentration and hemodilution are noted and briefly discussed.

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ACTIVITY OF DESOXYCORTICOSTERONE ACETATE IN PROPYLENE GLYCOL BY ORAL AND INTRAVENOUS ROUTES IN ADRENALECTOMIZED DOGS, AND ITS EFFECT ON THE CARDIAC ARHYTHMIA OF ADRENAL INSUFFICIENCY

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THE SUBLINGUAL ADMINISTRATION of desoxycorticosterone acetate in propylene glycol was introduced recently by Anderson, Haymaker and Henderson (1) in the treatment of Addison's disease. They found this method to be as effective as subcutaneous or intramuscular injections of the hormone in oil, while the ingestion of tablets of desoxycorticosterone acetate was of no value. It seemed desirable to test the effectiveness of this material in a laboratory animal. Adrenalectomized dogs were chosen for the investigation since a study had been made on the maintenance of such animals by injections of desoxycorticosterone acetate in oil and this would provide a basis for comparison (2). Oral administration of desoxycorticosterone acetate in propylene glycol was undertaken on dogs previously maintained by daily injections of hormone and the value of intravenous injections of the same material was investigated in dogs suffering severe adrenal insufficiency. Since our work was completed, a paper by Thorn et al. (3) has appeared. They confirm the activity of desoxycorticosterone acetate in propylene glycol, administered orally, in cases of Addison's disease and demonstrate its effectiveness in dogs.

TECHNIC

Five male dogs, adrenalectomized 3 weeks to 1.5 years previously and weighing 8 to 10 kg., were used in the oral tests. The methods used in the care of the animals and the chemical and other technics have been described previously (2). All of the dogs had been maintained by fairly large injections of desoxycorticosterone acetate in oil for at least a week prior to the initiation of the test of the propylene glycol preparation. They were well hydrated and in excellent condition as judged by activity, weight, non-protein nitrogen and packed red cell volume. The diet used was the same as in our previous work and consisted, essentially, of lean meat containing approximately 1 per cent of sodium chloride. The criteria of inadequate maintenance were a 50 per cent rise in non-protein nitrogen, a weight loss of 400 gm., and an increase in packed cell volume of more than 5 per cent. A significant change in two of these criteria was considered evidence of inadequate maintenance.

Three other adrenalectomized dogs were used for investigating the effectiveness of desoxycorticosterone acetate[2] in propylene glycol injected intravenously. These ani-

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[2] The desoxycorticosterone acetate in oil and in propylene glycol used in these experiments were generously supplied by Drs. Erwin Schwenk and Edward Henderson of the Schering Corp., Bloomfield, N. J.

mals also received a lean meat diet but with 2 per cent of sodium chloride. They were allowed to go into severe adrenal insufficiency, showing lethargy, anorexia, bradycardia and weakness, before treatment was instituted.

RESULTS

Effect of Oral Administration of Desoxycorticosterone Acetate in Propylene Glycol

Desoxycorticosterone acetate in propylene glycol, administered orally, was tested in three sets of experiments, two dogs being used in each. In the third test, one dog had been used in a previous experiment but had been adequately restored to health.

Experiment 1. Two dogs received 0.1 cc. (1 mg.) of desoxycorticosterone acetate in propylene glycol under the tongue 3 times daily at 3 to 4-hour intervals. This amount in milligrams is twice the maintenance dose of desoxycorticosterone acetate in oil which was administered intramuscularly (2). On the sixth day after the sublingual therapy was started, both dogs appeared lethargic and showed an increase in packed cell volume of 11 per cent (39 to 50 per cent), but little change in non-protein nitrogen. Each dog had lost over 600 gm. in weight. Although maintenance had not been effected, some evidence suggestive of hormonal activity was obtained, as adrenalectomized dogs on this diet usually develop severe insufficiency in less than 5 days following cessation of hormone injections.

TABLE 1. EFFECT OF ORAL TREATMENT OF ADRENALECTOMIZED DOGS WITH DESOXYCORTICOSTERONE ACETATE IN PROPYLENE GLYCOL. (DIET: 1 PER CENT NaCl)

	Treatment		Weight Loss	Packed Cell Volume		Non-Protein Nitrogen	
	Duration	Dosage		Initial	Final	Initial	Final
	days		*gm.*	% R.B.C.	% R.B.C.	*mg.* %	*mg.* %
Exp. 2							
Dog 1	10	1 mg., 6 times daily, under tongue	700	38	46	48	264
Dog 2	11	1 mg., 6 times daily, under tongue	800	41	47	43	56
Exp. 3							
Dog 1	10	2 mg., 3 times daily, in cheek	900	28	43	50	144[1]
Dog 3	10	2 mg. 3 times daily, in cheek	900	28	46	45	72[1]

[1] Following this investigation, daily intramuscular injections of 5 mg. of desoxycorticosterone acetate, in oil were given. At the end of 4 days the packed cell volume had fallen in *dog 1* to 39 per cent and in *dog 3* to 36 per cent, and the non-protein nitrogen had fallen to 80 and 57 mg. per cent, respectively.

Experiment 2. Two dogs received 0.1 cc. (1 mg.) of the propylene glycol preparation sublingually 6 times daily at intervals of at least one hour. This is 4 times the maintenance dose of desoxycorticosterone acetate by injection. Marked changes in weight, non-protein nitrogen and packed cell volume were evident in a week's time. The study was terminated on the tenth and eleventh days, respectively, due to the appearance of clinical signs of insufficiency in the two dogs, vomiting in one, and lethargy in the other. The weight loss from the start of the experiment, and the increase in non-protein nitrogen and packed cell volume are recorded in table 1. In the case of *dog 1*, the serum sodium had fallen from 141 to 131 m.eq. *Dog 2* showed no significant change in this respect. Following cessation of hormone injection on other occasions, both of these dogs went into a state of severe insufficiency in less than 4

days. Consequently, it would appear that some slight hormonal effect was exerted by the orally administered desoxycorticosterone acetate in propylene glycol.

Experiment 3. Two dogs received 0.2 cc. (2 mg.) of desoxycorticosterone acetate in propylene glycol 3 times daily, placed far back on the lower buccal surface of the cheek. The animals were exceptionally hydrated before the experiment was begun, as evidenced by unusually heavy weight and low packed cell volume. Refusal of food, vomiting or lethargy had not developed by the tenth day, although weight loss and blood changes were marked (table 1). At this time, desoxycorticosterone acetate in oil, 5 mg. a day injected intramuscularly, was substituted for the oral therapy. After 2 injections the non-protein nitrogen and packed cell volume had both fallen significantly, and after 4 injections these values approximated normal levels.

Cardiac arhythmias, which had been encountered in previous experiments (2), were not observed in any of the animals in this series. With the development of hemoconcentration, tachycardia developed, heart rates up to 160 per minute being recorded. This occurs infrequently in dogs on a 2 per cent sodium chloride diet either when they are developing adrenal insufficiency on submaintenance doses of cortical hormone or after cessation of hormone injections.

Effect of Intravenous Administration of Desoxycorticosterone Acetate in Propylene Glycol

The primary object of this part of the study was to ascertain whether dogs could be restored from severe adrenal insufficiency by the use of desoxycorticosterone acetate alone. Secondarily, we wished to determine whether it would correct the arhythmia encounted in adrenal insufficiency (2, 4, 5). The availability of a preparation which could be given intravenously made the investigation possible, since desoxycorticosterone acetate in oil injected intramuscularly is not absorbed fast enough to produce results in crisis.

In an earlier report (2) reference was made to the development of cardiac irregularities and cardiac malfunction leading to death in dogs which had been maintained on small doses of desoxycorticosterone acetate. On the basis of data available at that time, it seemed that desoxycorticosterone acetate lacked some factor necessary for efficient cardiac function. Results of subsequent experiments indicate that these findings were a manifestation of inadequate dosage insofar as the heart was concerned, rather than a complete lack of effect of this substance on cardiac metabolism.

A few words concerning the arhythmia of adrenal insufficiency are pertinent to the interpretation of the observations made in the present series. The arhythmia is a slow irregularity in which the bradycardia is clearly recognizable by auscultation, but the irregularity of the beats may be disclosed only by an electrocardiogram. Sometimes the arhythmia is inconstant. This is most likely to be true if it appears 6 or more hours before the animal becomes prostrate. The heart may take up a normal rate and rhythm spontaneously, or exercise, excitement or the administration of atropine may dispel the bradycardia and irregular rhythm. Usually the irregularity becomes more stable as the condition of the animal declines so that no influence other than specific measures for the treatment of adrenal insufficiency, such as intravenous hypertonic saline and/or cortical extract, will cause a return of normal rhythm.

During this investigation, therefore, it was imperative to establish evidence that the arhythmia had reached a state at which spontaneous recovery would not occur, and neither exercise nor excitement would lead to restoration of normal rhythm. It had been found that, if normal rate and rhythm were not restored by the administration of atropine, spontaneous recovery would not occur and that it could not be re-

established by exercise or excitement. Further, if atropine caused a restoration of normal rhythm in an animal in severe adrenal insufficiency, a subsequent return of arhythmia was resistant to any treatment except specific measures.

Desoxycorticosterone acetate in propylene glycol was administered intravenously in 1 and 2 cc. amounts, without dilution, to 3 dogs (*dogs 6, 7 and 8*) showing severe adrenal insufficiency. Two of the 3 animals were revived successfully.

Dog 6. Adrenalectomy was performed on May 16, 1941, and cortical extract therapy administered until June 19. At that time the resting heart rate was 92 per minute. Six days later the heart rate was periodically slow and irregular, and the dog vomited. Twenty-four hours later the animal was lethargic and weak; by femoral puncture the blood pressure was 63 mm. Hg at 9:00 A.M., and the heart rate was 60 per minute. An electrocardiogram at this time showed absent P waves. At 9:50 A.M. a blood volume study was commenced, 32 cc. of blood being removed in the next 100 minutes. At 11:50 A.M. the heart rate was 52 per minute and irregular; 20 mg. of desoxycorticosterone acetate in propylene glycol was given intravenously. Two minutes later the dog vomited and seemed much weaker. Death occurred 25 minutes after the injection. At postmortem examination, characteristic intense congestion of the mucosa of stomach and small intestine, pancreas, kidneys and liver was found. The heart was in diastole but showed no other abnormality in the gross. No adrenal tissue was found.

Dog 7. Adrenalectomy was performed on Feb. 21, 1941. The animal was maintained by cortical extract and then used for assay of an adrenal cortex preparation. On June 25, while receiving a submaintenance dose of extract, the dog left a little food uneaten. The next day, half the food ration was untouched. The following morning, June 27, at 9:00 A.M. the heart rate was found to be 52, having declined from the normal range of 80 to 90 beats per minute. The packed cell volume had risen from 49 per cent on June 17 to 56 per cent. An electrocardiogram showed absent P waves and a grossly irregular ventricular rhythm. By 11:00 A.M the heart rate had declined to 40 per minute and an electrocardiogram showed an idioventricular rhythm. At 11:30 A.M. the heart rate was unchanged and the blood pressure, by femoral puncture, was 82 mm. Hg. At this time 2.5 mg. of atropine sulphate was injected intravenously. Within 30 seconds the heart rate had increased to 70, but this gradually declined so that at the end of 80 minutes it was 54 per minute. Acceleration of rate following atropine administration, without return of normal rhythm, has been observed frequently. Ten minutes later, at 1:00 P.M., 20 mg. of desoxycorticosterone acetate in propylene glycol was given intravenously. Forty minutes later the heart was auscultated and found to be beating regularly at a rate of 120 per minute. Thirty-five minutes later, at 2:15 P.M., the rate was the same and an electrocardiogram showed a normal rhythm. Another intravenous injection of 10 mg. of desoxycorticosterone acetate in propylene glycol was given at this time. At 5:00 P.M. the heart rate was still fast and regular. A sample of blood taken at this time showed a packed cell volume of 55 per cent. Food and water, which had been withheld all day, was now given and next morning the food had been eaten and the water consumed. The heart rate was 120 per minute and regular. Subcutaneous injections of desoxycorticosterone acetate were commenced for maintenance. The animal is still well and vigorous.

Dog 8. Adrenalectomy was performed on June 6, 1941. Daily subcutaneous injections of desoxycorticosterone acetate in oil were used to maintain the dog until June 19, when the packed cell volume was 41 per cent. On June 30, no food was eaten and the heart rate, which had been about 96, declined between 3:00 and 4:30 P.M. to 52 beats per minute. An electrocardiogram showed that this was due to many dropped beats. Exercise accelerated the heart rate to 108 at this time but by 5:45 P.M. it had declined to 60 per minute, again due to dropped beats. Femoral blood pressure was recorded at 105 mm. Hg. Administration of 3 mg. of atropine sulphate increased the heart rate from 60 to 156 per minute. Blood obtained at 6:10 P.M. showed a packed cell volume of 60 per cent and serum potassium of 7.4 m.eq. By 8:00 P.M. the heart rate was still high (120 per minute) but the blood pressure had declined to 85 mm. Hg and the dog looked ill and was very weak. At 11:30 P.M. the heart rate was still 120 and the packed cell volume 61 per cent. One hour later the heart rate had fallen to 66 per minute and the dog was barely able to stand. Half an hour later (1:00 A.M., July 1) the heart rate was slower, 48 beats per minute. Desoxycorticosterone acetate in propylene glycol,

20 mg., was given intravenously. During the next 40 minutes the heart rate was practically unchanged; a further intravenous injection of 10 mg. of desoxycorticosterone acetate in propylene glycol was given. One hour later, at 2:40 A.M., the heart rate was the same; the 10 mg. dose of desoxycorticosterone acetate by intravenous injection was repeated. At 3:10 A.M. the heart rate was 60 per minute and at 3:30 A.M. it was 62, with groups of 3 or 4 fast beats. At 4:05 A.M. the heart rate was 110 per minute and regular, and the dog showed more interest in his surroundings. No food or fluid had been allowed since 4:00 P.M. the previous day. At 4:15 A.M. 5 mg. of desoxycorticosterone acetate in oil was given subcutaneously. At 5:00 A.M. and again at 11:00 A.M. the heart rate was still 110 per minute and regular. Subcutaneous injections of desoxycorticosterone acetate in oil were continued for maintenance. The dog was alive 3 months later.

DISCUSSION

In the experiments in which desoxycorticosterone acetate in propylene glycol was put under the tongue or in the cheek, the animals swallowed an undetermined amount. Consequently, the results are not comparable to those obtained in patients who coöperate by allowing absorption to take place in the mouth. It may be said, however, that under the conditions of our experiments the oral administration of small amounts of propylene glycol containing as much as 2 mg. of desoxycorticosterone acetate 3 times daily, does not effectively maintain adrenalectomized dogs. It is known from previous experience that injection of 1.25 to 1.87 mg. of desoxycorticosterone acetate in oil daily will maintain such animals on the 1 per cent sodium chloride diet. This confirms the observation of Kuizenga, Nelson and Cartland (6) that desoxycorticosterone acetate orally is much less effective than when administered parenterally.

In a recent paper, Thorn et al. (3), 1941, have demonstrated that desoxycorticosterone acetate in propylene glycol will maintain adrenalectomized dogs when used in a dosage 3 to 4 times as great as that intramuscularly. In their experiments the dog's mouth was held tightly closed for one minute after administration of the sublingual dose. This procedure appears to have permitted much greater absorption of the desoxycorticosterone acetate than was obtained in our experiments.

Cardiac irregularities were absent and tachycardia present in adrenal insufficiency in dogs receiving desoxycorticosterone acetate orally. This might be attributed to the fact that these animals had been treated with relatively large amounts of hormone and were well hydrated before injections were stopped and the ineffectual oral therapy started. Presumably the heart of a dog previously treated with large amounts of hormone has a greater capacity for acceleration and less tendency to arhythmia because the supply of hormone, or its influence, in the heart is not so soon exhausted as in dogs maintained on a high salt-low hormone regimen. Animals receiving larger amounts of salt in the diet, and consequently requiring less desoxycorticosterone acetate or cortical extract for maintenance, may develop cardiac irregularities during the maintenance period (2). The heart rarely accelerates much after the injections of hormone are stopped (*dogs 6*, 7 and 8 are examples), and cardiac irregularities occur frequently with the development of adrenal insufficiency. These dogs were not moribund when cardiac irregularities developed. The blood pressure in *dog 7* was 82 mm. Hg when the arhythmia had been present at least 2.5 hours, and in *dog 8* the pressure was 105 mm. Hg more than an hour after the irregularity was observed.

The successful outcome attending treatment of 2 of the 3 animals with intravenous injections of desoxycorticosterone acetate in propylene glycol is of both practical and theoretical interest. It provides evidence that this preparation is a safe and effective form of treatment for dogs in severe adrenal insufficiency. It also shows that the cardiac arhythmia of adrenal insufficiency in dogs can be corrected by this agent

alone, thus refuting the suggestion previously advanced (2) that desoxycorticosterone acetate lacks some factor necessary for cardiac metabolism. Doubtless desoxycorticosterone acetate in propylene glycol would be even more effective combined with other intravenous fluids, as we (4) have found that hypertonic glucose or saline leads to a restoration of normal rhythm in animals suffering from adrenal insufficiency in a matter of seconds or minutes. Re-establishment of normal cardiac rhythm is an important feature in the recovery of dogs which suffer from marked bradycardia in adrenal insufficiency.

As judged by its ability to restore abnormal cardiac rhythm, the rapidity of action of desoxycorticosterone acetate in propylene glycol administered intravenously has been found to be considerably greater than that of aqueous cortical extract administered in a similar manner. Normal cardiac rhythm is restored by desoxycorticosterone acetate in propylene glycol too rapidly to be the result of an influence on urinary excretion of potassium. It may be unrelated to restoration of blood volume also for, in *dog* 7, only a small degree of dilution had taken place at the time the bradycardia disappeared. The decrease in volume of packed red blood cells was more marked at the time of the assumption of a faster rate in *dog* 8. In this connection, Muntwyler and his associates (7) minimize the importance of the correction of water and electrolyte changes by cortical extract in restoring dogs in adrenal insufficiency. They are of the opinion that such a correction is secondary in importance. Our results indicate that improvement in cardiac function probably is of primary importance.

Circumstances prevented the performance of extensive blood studies in the last 3 animals. The elevated serum potassium in *dog* 8 was characteristic of adrenal insufficiency, as were the weight loss, anorexia, weakness, increase in packed cell volume, hypotension and bradycardia observed in all 3 dogs. The animal that died (*dog* 6) had had some 32 cc. of blood withdrawn during the blood volume study in adrenal insufficiency. This blood loss probably contributed to the failure of desoxycorticosterone acetate in propylene glycol to effect recovery.

SUMMARY

Desoxycorticosterone acetate dissolved in propylene glycol, administered orally, did not effectively maintain adrenalectomized dogs. Evidence that some absorption of the active material took place is suggested by the failure of severe adrenal insufficiency to develop before the end of 10 days' treatment.

Desoxycorticosterone acetate administered to 3 dogs in severe adrenal insufficiency restored two. In these 2, the intravenous administration of desoxycorticosterone acetate in propylene glycol was followed by restoration of normal cardiac rhythm and by improvement in the general condition of the animals within about 3 hours.

The opinion formerly advanced that desoxycorticosterone acetate lacks some factor necessary for cardiac metabolism is not substantiated by these later experiments.

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NEW COLOR REACTION FOR CERTAIN URINARY 17-KETOSTEROIDS[1]

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THE ZIMMERMAN color reaction has been extensively applied to urinary extracts for the quantitative determination of urinary 17-ketosteroids. It has the disadvantage that chromogens other than 17-ketosteroids react with the Zimmerman reagent. The non-ketonic chromogens of the neutral urinary extracts may be discounted by the use of a color correction equation (1, 2) or by direct chemical removal with measurement only of the ketonic extracts (3, 4). If the non-ketonic chromogens vary qualitatively in their reaction with the Zimmerman reagent then the value of applying a color correction equation is questionable. Among the neutral ketonic steroids certain ketones that may be present give atypical colors; thus 3-ketosteroids give a yellow color and 20-ketosteroids a red color clearly different from the typical pink color of the 17-ketosteroids (4, 5, 6). Compounds having absorption spectra like those of 3-ketosteroids appear frequently in the non-alcoholic neutral urinary extracts (4), and 20-ketosteroids are typical, particularly of pregnancy urines in which they may be present in amounts much exceeding the 17-ketosteroids, contributing inordinately to the color titer (7).

A color reaction having greater specificity than the Zimmerman reaction should, therefore, have distinct advantages. In this paper we describe the reaction of antimony trichloride with a number of steroid compounds. Under suitable conditions a blue color can be developed with certain 17-ketosteroids and with no other of a series of ketonic steroids known to occur in neutral urine extracts. The applicability of this color reaction to urine extracts is also demonstrated.

MATERIAL AND METHODS

A highly concentrated solution of antimony trichloride (c.p.) in a mixture of 9 parts of glacial acetic acid to one part acetic anhydride (c.p.) is prepared. For standard determinations we have used 3.8 gm. $SbCl_3$ to 1 cc. of the acid-anhydride mixture. To a dried steroid (or urine extract) preparation in a test tube, 0.2 cc. of the reagent is added drop by drop from a microburette, and the stoppered solution is heated in a boiling water bath for 20 minutes. Within ½ to 1 minute after immersing the test tube in the water bath the tube is thoroughly shaken to insure complete solution of the steroid preparation. The tube is kept stoppered to prevent the entrance of moisture. After heating, the tube is cooled and the solution diluted with glacial acetic acid. The diluent is added slowly with shaking. The typical color develops on dilution, and reaches a maximum some time after dilution. With the solution of 3.8 gm. of $SbCl_3$ to 1 cc. of the acid-anhydride mixture the maximum usually is attained in 40 to 60 minutes at room temperature.

The pure steroids tested in the reaction were divided into three groups: *a*) alcoholic ketones, including testosterone, androsterone, isoandrosterone, etiocholanol-3α-17one, androstanol-3β-(?)one, dehydroisoandrosterone, allopregnanolone, and epi-

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pregnanolone; *b*) non-alcoholic ketones, including androsterone-17, androstenedione, progesterone, and cholestenone; *c*) non-ketonic steroids, including androstanediol, pregnandiol, and cholesterol. In addition, we have employed androsterone sulfate, androsterone succinate and dehydroiso-androsterone succinate. Unless otherwise noted, the results are expressed in terms of 100 micrograms of steroid. The urinary extracts employed are described in the text.

The spectrophotometric measurements were made with a Coleman Universal

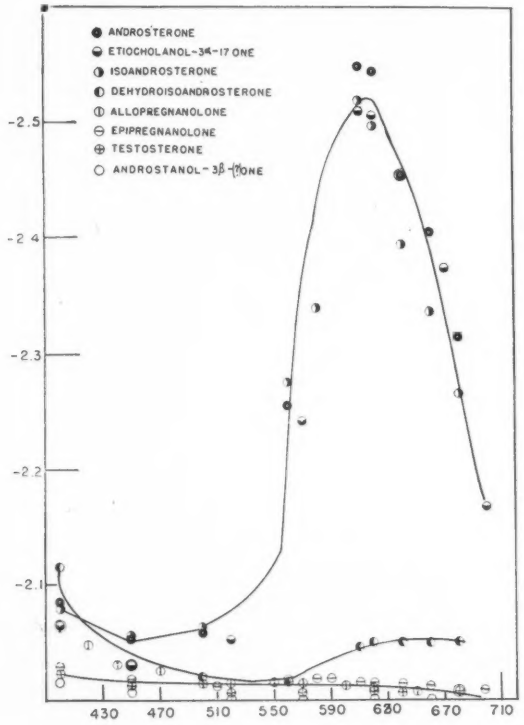

Fig. 1. Absorption spectra of various alcoholic ketonic steroids (100 μg. amounts) in the $SbCl_3$ reaction. Ordinate, absorption (log 1/transmission); abscissa, wave length in millimicra.

Spectrophotometer. The band width of the monochromator of this instrument at any wave length is 30 to 35 millimicra so that the absorption measurements are monochromatic only within these limits.

RESULTS

Crystalline steroids. In figures 1 to 3 are presented the absorption spectra of the colors developed by the three groups of pure steroids employed. It should be noted *a*), that of the alcoholic ketosteroids only androsterone and its isomers, isoandrosterone and etiocholanol-3α-17one, develop an intense blue color having an absorption maximum in the region of 610–620 millimicra; *b*), that dehydroisoandrosterone develops a faint bluish color having a considerable yellowish tinge due to the absorption in the region of 400 μμ; *c*), that the other alcoholic ketosteroids tested develop a faint

yellowish color which has an absorption at 610–620 μμ varying from 0 to less than 2 per cent of the androsterone value; *d*), that of the non-alcoholic ketosteroids only androstenone-17 develops in equal intensity the blue color typical of androsterone and its isomers; *e*), that the other non-alcoholic ketosteroids tested develop negligible coloration; *f*), that androstanediol develops the typical blue color but in an intensity equal to about 17 per cent of that developed by androsterone; *g*), that pregnandiol develops a blue color having an absorption maximum at 680–690 μμ; *h*), that cholesterol

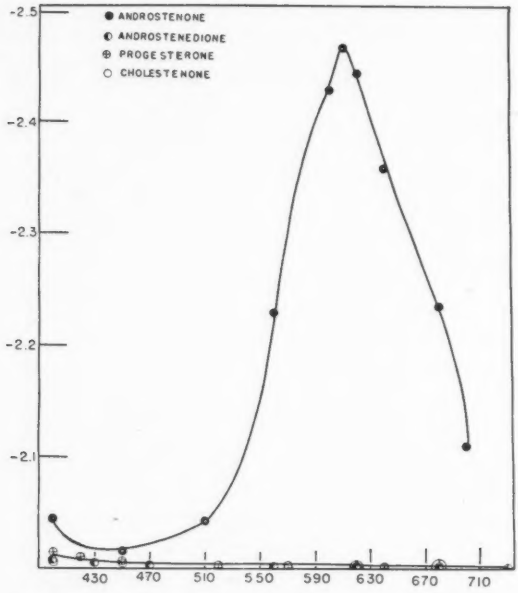

Fig. 2. Absorption spectra of various non-alcoholic ketonic steroids (100 microgram amounts) in the $SbCl_3$ reaction. Ordinate, absorption; abscissa, wave length.

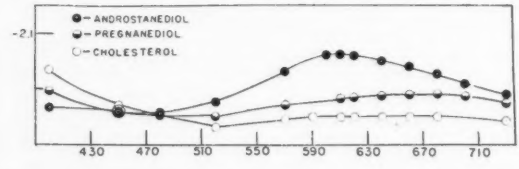

Fig. 3. Absorption spectra of various non-ketonic steroids (100 microgram amounts) in the $SbCl_3$ reaction. Ordinate, absorption; abscissa, wave length.

develops a faint color having an absorption maximum at 400 μμ and a second, lesser maximum at 680–690 μμ.

In figure 4 are presented the absorption spectra of the three androgen esters tested. The data demonstrate that the esters develop the same color in intensity equal to that of the free steroids. The absorption curve of androsterone sulfate is shifted somewhat to the left; this may be due to the presence of minor impurities since this material was purified from urine extracts.

Urine extracts. The absorption spectra of the alcoholic ketonic neutral urine extracts from three types of urine are presented in figure 5. The maxima are adjusted to coincide with the extinction value obtained with approximately 120 μg. of androsterone. It is clear that the urine extracts have higher extinction coefficients than the pure steroid over the range 400 to 530 μμ. The specimen taken at labor and delivery shows the greatest deviation from the pure androsterone curve. By the application of the Zimmerman reaction, we (7) found this specimen to contain approximately 4 mg. of 20-ketosteroid to each mg. of 17-ketosteroid. This should, with the $SbCl_3$ reaction give an absorption at 610 to 620 μμ of 20-ketosteroid equal to approximately 8 per cent of the absorption due to 17-ketosteroid. By applying the color correction equation of Fraser, et al. (1), one can correct roughly for the 20-ketosteroid content of this urine extract on the basis of the absorption at 400 μμ, assuming that all of the extra absorption at 400 μμ is due to 20-ketosteroid. The result

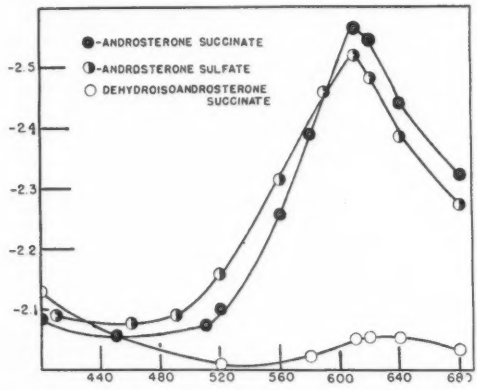

Fig. 4. Absorption spectra of certain esters of androsterone and dehydroisoandrosterone. 100 micrograms equivalent of the free compounds. Ordinate, absorption; abscissa, wave length.

of such calculation indicates that 9 per cent of the absorption at 610 to 620 μμ is due to 20-ketosteroid. In the case of the other two urine extracts the overestimate calculated on this basis obviously becomes less. In the case of urine from the normal male the use of the color correction equation on the assumption that dehydroisoandrosterone contributes the extra absorption at 400, gives the result that 17 per cent of the absorption at 610 to 620 μμ is due to this compound. Since there are undoubtedly other materials present reacting to give absorption at 400 μμ, this estimate represents a maximum.

The data of figure 5 are corrected for the intrinsic color of the sample by the taking of readings on an aliquot to which was added the acetic acid-acetic anhydride mixture with no $SbCl_3$. In the most highly pigmented sample (labor and delivery urine), the intrinsic color was responsible for less than 1 per cent of the color developed at the maximum. This indicates a special advantage of the $SbCl_3$ reaction; in the region of maximum absorption intrinsic extract colors, which are usually browns or yellows, show little or no absorption.

Special conditions affecting the color reaction. The development of the typical color as the result of a reaction between $SbCl_3$ and the steroids tested depends on *a*) the concentration of $SbCl_3$, *b*) the time of heating, *c*) the nature of the diluent.

The use of a highly concentrated solution, or of amorphous $SbCl_3$ seems to be

mandatory. Thus no color developed after heating androsterone for the usual time when the $SbCl_3$ concentration was 1 gm. per cc. If a saturated solution of $SbCl_3$ in chloroform is used to take up the dried steroid and the chloroform is evaporated off, the amorphous $SbCl_3$ remaining will react with the steroid and on dilution with acetic acid the typical color will develop (fig. 6). The presence of more than a limited amount of solvent, therefore, interferes with the initial reaction.

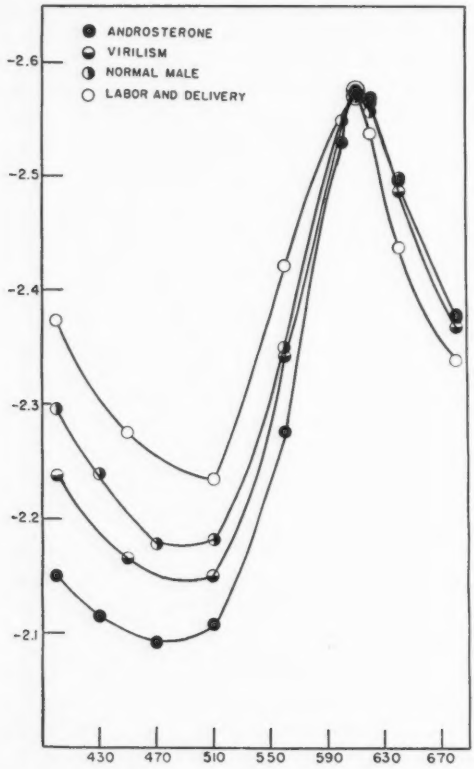

Fig. 5. ABSORPTION SPECTRA OF THE ALCOHOLIC KETONIC FRACTIONS of the urinary extracts compared with the absorption spectrum of pure androsterone in the $SbCl_3$ reaction. The urinary extract data have been adjusted by coincidence of maxima at 610 mm. at a level equivalent to that obtained with 118 of androsterone. Ordinate, absorption; abscissa, wave length.

We have examined the effect of placing the reacting mixture for various times in the boiling waterbath. The intensity of the developed color will increase with increasing periods of heating up to 12 minutes, with no further increase for heating periods from 12 to 20 minutes. A typical series using 100 μg. is as follows: for heating periods of 3, 8, 12, 16 and 20 minutes the extinction coefficients at 610 $\mu\mu$ were 0.275, 0.371, 0.515, 0.505, 0.520, respectively when glacial acetic acid was used as the diluent. We have adopted 20 minutes as a standard heating period to insure effective reaction.

The chief variations in the color reaction appear to be due to the dilution medium.

We have employed chloroform, acetic anhydride and glacial acetic acid diluted with various amounts of water.

When chloroform is used as a diluent special care must be taken to avoid moisture; cloudy solutions often develop with the absorption of H_2O. The rate of color

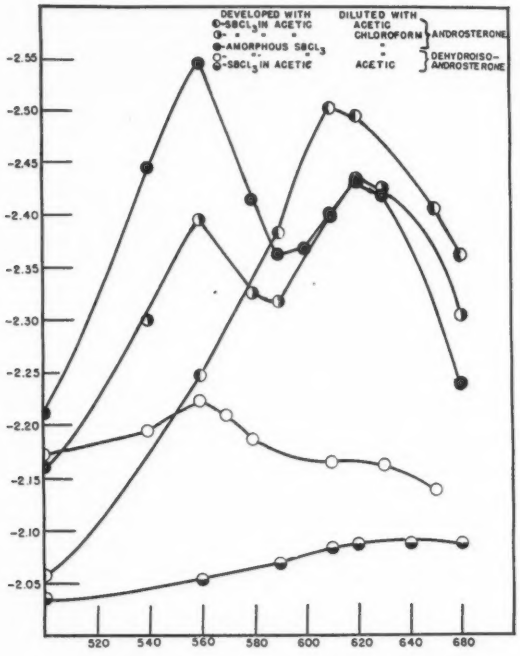

Fig. 6. Absorption spectra of the colored compounds obtained after treatment of androsterone and dehydroisoandrosterone with $SbCl_3$ under various conditions. 80 microgram quantities of steroid were employed. Note that the color developed upon diluting the reaction mixture with chloroform has two absorption maxima, whereas glacial acetic acid dilution yields a color having a single absorption peak. Ordinate, absorption; abscissa, wave length.

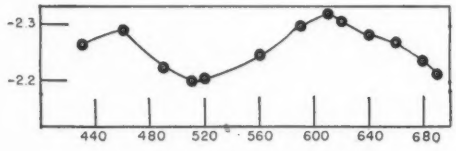

Fig. 7. Absorption spectrum of the colored compound developed by androsterone when the $SbCl_3$ reaction mixture is diluted with acetic anhydride. Ordinate, absorption; abscissa, wave length.

development is slower. Dehydroisoandrosterone develops the same color as androsterone but is less intense (fig. 6). Finally, the color developed in chloroform shows two absorption maxima instead of the single peak obtained when acetic acid is used as diluent. This is illustrated in figure 6.

When acetic anhydride is used as diluent the color that androsterone develops is a green with absorption maxima at 460 and 610 $\mu\mu$ and a rather high general absorp-

tion (fig. 7). The absorption at 610 $\mu\mu$ is less than that obtained with androsterone when the color is developed with glacial acetic acid.

When glacial acetic acid is used as the diluent Beer's law is not obeyed. This is illustrated in table 1, column 2, which demonstrates that the lower concentrations of androsterone give lower extinction coefficients. This is due to the greater rate of fading of the more dilute solutions and a shift with fading in the absorption maximum from 610 $\mu\mu$ to higher wave lengths. This is demonstrated in figure 8. The color can be stabilized and Beer's law obeyed by adding H_2O to 5 per cent to the acetic acid

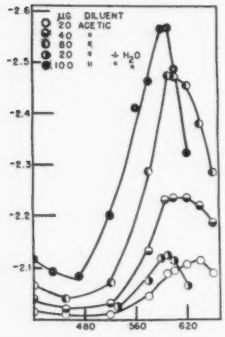

Fig. 8. Shift of absorption peak with dilute androsterone solutions in the $SbCl_3$ reaction when glacial acetic acid is used as diluent. Note that when the diluent is 95 parts glacial acetic acid: 5 parts distilled H_2O the absorption maximum does not shift. Ordinate, absorption; abscissa, wave length.

diluent (table 1, column 3). The dilute samples give the same absorption spectra as the concentrated samples, indicating no fading to an atypical color (fig. 8).

Using the diluted acetic acid as diluent, we have been unable to detect any significant differences in extinction coefficients when incubation is carried on in diffuse light or in the dark; direct sunlight, however, caused a reduction of approximately 20 per cent in the value.

Table 1. Extinction coefficients (100 micrograms of androsterone equivalent) using various quantities of androsterone with concentrated and diluted acetic acid as diluents

(1) Amount of Androsterone μg.	Diluent (2) Glacial acetic acid	Diluent (3) Glacial acetic acid 95 parts:H_2O 5 parts
10	0.400	
20	0.445	0.548
33		0.540
40	0.562	0.528
80	0.590	
160	0.582	0.542
197		0.550
200	0.544	0.554

Comparison of Zimmerman color titers with the antimony trichloride reaction titers. The urines of two groups of male and female hospital patients were pooled separately and the ketonic neutral fractions were obtained from one-liter aliquots of each using the micro-Girard separation previously described (4). One-half of each ketonic fraction was subjected to further separation into alcoholic and non-alcoholic fractions by succinic anhydride treatment (4). The titers of the total ketonic fraction and of the alcoholic and non-alcoholic fractions were determined by the application of *a*) the Zimmerman reaction and *b*) the antimony trichloride reaction. The resultant data are presented in table 2.

These data demonstrate *a*) that the Zimmerman titer is higher than the $SbCl_3$ color titer, *b*) that the titer difference by the two methods is least in the alcoholic ketonic fractions, greatest in the non-alcoholic fractions, and intermediate in the total ketonic fractions.

TABLE 2. 17-KETOSTEROID TITERS OF VARIOUS NEUTRAL FRACTIONS OF POOLED URINES FROM HOSPITAL PATIENTS AS DETERMINED BY THE ZIMMERMAN REACTION AND THE ANTIMONY TRICHLORIDE REACTION

Urine From	(a) Zimmerman Titers			(b) Antimony Trichloride Titers			(b) as % of (a)		
	Total ketonic	Alcoholic ketonic	Non-alcoholic ketonic	Total ketonic	Alcoholic ketonic	Non-alcoholic ketonic	Total ketonic	Alcoholic ketonic	Non-alcoholic ketonic
	mg. 17-ketosteroid per liter			*mg. androsterone equiv. per liter*					
Men I	6.47	3.51	1.88	4.24	3.00	1.01	65.6	85.5	53.7
Women I	5.60	2.64	1.54	3.81	2.57	0.99	68.1	97.3	64.3
Men II	7.90	5.11	2.44	5.21	4.14	1.25	66.8	81.0	51.2
Women II	2.70	1.11	0.81	1.62	1.02	0.42	60.0	92.0	51.6

DISCUSSION

Concerning the technique of the color reaction described, it is clear that a highly concentrated reacting solution of $SbCl_3$ is required for any color development and that the nature of the color developed depends upon the nature of the diluent employed. It is therefore necessary to control rigidly these conditions. We now routinely follow the conditions laid down in the section on methods, but for color stability the reaction mixture should be diluted with a solution containing 95 parts of glacial acetic acid to 5 parts of water. To avoid any possible bleaching by light, the diluted samples are placed in the dark in a constant-temperature water bath.

The data on the pooled urine samples confirm the expectation induced by the specificity of the color reaction. The principle 17-ketosteroids of human urines are alcoholic ketones, androsterone and its isomers and dehydroisoandrosterone. The latter compound, which reacts only feebly in the $SbCl_3$ reaction under our standard conditions, in normal human urines is present in only small amount. According to the isolation studies of Callow and Callow (8, 9) the dehydroisoandrosterone content of human urines is 0.1 to 0.2 mg. per liter, whereas androsterone and its α-isomer amount to about 3.0 mg. per liter (see also Hirschmann, 10). Baumann and Metzger (11) find the Zimmerman color titer of the α-steroids equal to 85 to 98 per cent of the total color titer. Since the β-steroids include isoandrosterone as well as dehydroisoandrosterone (12, 13) it is obvious that the minor contribution of the latter to the $SbCl_3$ color titer cannot account for the differences presented in table 2. Only in the alcoholic ketonic fractions of the women's urines are the differences sufficiently small to be possibly accountable to the minor contribution of dehydroisoandrosterone to the $SbCl_3$ color titer.

The data of table 2 demonstrate that materials reacting with $SbCl_3$ in the non-alcoholic fractions are equivalent to 51 to 64 per cent of the Zimmerman color titer. We have previously noted atypical Zimmerman colors in this fraction (4). The non-alcoholic 17-ketosteroids which have been isolated from human urines are androstenone-17 (12, 13, 14) and $\Delta^{3,5}$ androstadieneone-17 (13). The former gives the typical color in the $SbCl_3$ reaction (fig. 2), the latter we have been unable to test, but it is apparently present in small amount and, by analogy, we should expect it to react like dehydroisoandrosterone. It would seem profitable, therefore, to investigate the Zimmerman chromogen of this fraction which does not react with $SbCl_3$.

SUMMARY

An intense blue color (absorption maximum at 610 $\mu\mu$) is developed by androsterone and its isomers and by androstenone-17 after heating with concentrated $SbCl_3$ solution (or amorphous $SbCl_3$) and dilution with glacial acetic acid. Androsterone succinate and androsterone sulfate develop the same intense color. Androstanediol develops qualitatively the same color but in reduced intensity. Dehydroisoandrosterone and its succinate develop a weak yellowish-blue color and pregnandiol a weaker, somewhat similar color. Testosterone, allopregnanolone, epipregnanolone, androstenedione, androstanol-3β-(?)one, progesterone, cholestenone, and cholesterol develop extremely weak yellow colors. The optimal method for development of the typical stable color requires *a*) using a solution containing 3.8 gm. of $SbCl_3$ per 0.9 cc. of glacial acetic acid and 0.1 cc. of acetic anhydride which is added drop by drop in 0.2 cc. to the dried steroid preparation, *b*) heating, with shaking for 20 minutes in a boiling waterbath, *c*) diluting with a solution containing 95 parts of glacial acetic acid to 5 parts of distilled water, *d*) incubating in the dark at room temperature for 40 to 60 minutes.

When chloroform is used as the diluent, a qualitatively different color develops with androsterone, having absorption maxima at 560 and 620 $\mu\mu$. Dehydroisoandrosterone with chloroform dilution develops a similar, less intense color. Androsterone with acetic anhydride dilution develops a greenish color with absorption maxima at 460 and 610 $\mu\mu$.

The application of the color reaction to urinary neutral ketonic extracts from the urines of men and women gives a color titer less than that of the Zimmerman titer, the largest difference (36 to 49 per cent less) occurring in the non-alcoholic ketonic fractions.

The writer is indebted to Mr. Irving Weiss for valued technical assistance. Dr. W. H. Pearlman contributed a number of the pure steroids employed. Dr. E. Schwenk of the Schering Corporation supplied the progesterone and testosterone, Dr. E. Venning the androsterone sulfate, Dr. H. Hirschmann the etiocholanol-3α-one-17, Dr. Ralph Dorfman the androstanediol, Dr. R. D. H. Heard the androstanol-3β-(?) one.

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EFFECT OF SULFONAMIDES AND THIOUREAS ON THE THYROID GLAND AND BASAL METABOLISM[1,2]

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IN A PRELIMINARY paper (1) in 1941 we reported that sulfaguanidine, a sulfonamide employed to combat intestinal infections, produced a remarkable enlargement of the thyroid gland in rats, either in the presence or absence of para-aminobenzoic acid. The widespread use of sulfonamides,[3] and the certainty of their ever-extending use in both therapy and prophylaxis, demanded an immediate and detailed investigation of this effect on the thyroid gland (and possibly on the basal metabolic rate) of the rat and other experimental animals to provide a basis for the search of a similar reaction in man. Furthermore, since this effect was not prevented in the rat by para-aminobenzoic acid, and therefore did not parallel the bacteriostatic activity of sulfaguanidine, the possibility of elucidating other biological reactions, and hence new therapeutic uses for sulfonamides, could not be overlooked.

Our subsequent experiments disclosed that the thyroid enlargement was not effected by sulfanilic acid or guanidine, but that it was produced by a series of sulfonamides and thioureas. The thyroid hypertrophy was accompanied by a marked drop in the basal metabolic rate. Both of these changes were prevented or cured by administering thyroxin,[4] but not by increasing the iodine content of the diet (2).

Richter and Clisby (3) have recently reported thyroid enlargement associated with loss of weight and other toxic reactions in rats fed phenyl thiourea in their drinking water.

In attempting to isolate the goitrogenic principle present in rape seed, the possibility of its being a derivative of thiourea was tested by Kennedy (4, 5). He found that oral administration of small amounts of allyl thiourea to rats caused not only thyroid enlargement but also changes in the anterior pituitary. These changes in the pituitary are described as similar to those found in rats fed rape seed (6). The results obtained with allyl thiourea are in agreement with our observations, with respect to both the thyroid hyperplasia and alteration of the cellular pattern of the anterior pituitary.

The present communication is a detailed report and an extension of our previous observations; following a description of methods, it is divided into five sections. *a*) Biometric and histologic data on the thyroids of rats of different ages fed several levels of sulfaguanidine over varying periods of time; the action of other sulfonamides, thioureas and other compounds on the thyroid of the rat; the effect in other species. *b*) The change in the basal metabolic rate. *c*) The prevention of the thyroid-metabolic changes. *d*) The cellular changes in the anterior pituitary. *e*) A discussion of the possible mechanisms involved, together with the results of pertinent experiments.

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[2] Supported by a grant from the Rockefeller Foundation.
[3] Sulfonamides kindly supplied by: Lederle Laboratories, Inc., Pearl River, N. Y., Sharp and Dohme Inc., Glenolden, Pa. Parke, Davis and Co., Detroit, Mich., Merck and Co., Inc., Rahway, N. J.
[4] Thyroxin supplied by Roche-Organon, Inc., Nutley, N. J.

METHODS AND RESULTS

Soon after our preliminary report in which purified diets were employed, it was found that thyroid enlargement could be obtained equally well in rats receiving the McCollum stock diet[5] plus the drugs. This diet was employed for experiments with rats and mice. Guinea pigs were fed a mixture composed of equal parts of the McCollum stock diet and ground rabbit pellets.[6] Chickens were maintained on a commercial mash.[7] Dogs were fed a mixture of ground meat, milk powder, yeast and cod liver oil. Except in the last instance, in which the drugs were mixed by hand with the ground meat, a uniform distribution of the drugs was insured by the aid of a mechanical rotary mixer. The rats and mice received distilled water, which contained on one day each week 20 mg. of iodine and 40 mg. of potassium iodide per liter, while the other animals received tap water daily. All animals were housed in metal cages. At the end of an experiment the animals were killed with chloroform, the thyroid dissected out and immediately weighed on a torsion balance. In a large series of rats the thyroid was removed intact on the trachea and immediately dropped into Zenker-formol, which was used as the fixative for all tissues. The thyroid and other tissues were embedded in paraffin and stained with hematoxylin and eosin. Since this latter stain did not give adequate cellular definition of the pituitary, Altmann's aniline acid fuschin, and methyl green for differentiation of the acidophils was used, and Mallory II for the differentiation of the basophils.

Free and total sulfonamides were determined by the method of Marshall and his colleagues (7, 8). Figures for sulfaguanidine are reported in terms of the anhydrous compound. The drug levels reported on the rat probably represent minimal concentrations as the blood samples were taken between 10:00 A.M. and 12:00 noon. Marshall, Litchfield and White have shown this to be true in the case of the mouse (8). Blood was taken directly from the heart when the animal was to be killed, or from the tail vein when the animal was to be maintained on the experimental regimen. Hemoglobin levels were determined as acid hematin.

The basal metabolic rate in rats was determined first in the spirometer designed by Tainter and Rytand (9)[8] and later, in the case of the thyroidectomized animals, in the apparatus of Benedict (10).[9] All rats were starved for 20 hours before the basal metabolic rate was determined and several preliminary runs were made at weekly intervals to accustom the animals to the apparatus. The temperature was kept at or near 28° C. throughout the measurements. The surface area was estimated by the following formula: $S = 10w^{2/3}$ (11).

Thyroid Enlargement

Three groups of rats selected according to size and age were used in our experiments; immature rats weighing from 35 to 55 gm. and 21 to 28 days of age, young actively growing adult rats, and female rats past the period of active growth, one year of age or older.

Immature rats. A greater part of the biometric and histologic data on the thyroid was obtained from immature animals fed 2 per cent of sulfaguanidine for from 2 to 55 days, or fed varying levels of the drug, 0.5 to 3.0 per cent, for 14 days.

[5] Wheat 20.0; maize 20.0; rolled oats 20.0; flaxseed oil meal 10.0; crude casein 3.5; whole milk powder 25.0; $CaCO_3$ 0.5; NaCl 1.0; ferric citrate 50 gm. per 100 lb.; cupric sulphate 20 gm. per 100 lb.; Ca:P ratio 1.1:1.

[6] B-B Laboratory Rabbit Diet, Feed A. Maritime Milling Co., Inc., Buffalo, N. Y.

[7] Chicken Purina Growena Chow, Ralston Food Products, St. Louis, Mo.

[8] We are indebted to Dr. E. K. Marshall, Jr., of the Johns Hopkins Medical School for the use of two metabolism chambers (designed according to Tainter and Rytand).

[9] The Benedict one-unit rat metabolism apparatus was loaned to us through the courtesy of Dr. Thorne M. Carpenter of the Carnegie Institution of Washington, Nutrition Laboratory, Boston, Mass.

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Hyperemia of the thyroid, an invariable reaction to a drug which caused thyroid alteration, was readily detected in immature rats after 3 or 4 days on the diet containing 2 per cent sulfaguanidine. In 12 pairs of littermate rats, matched according to body weight and sex, the feeding of 2 per cent of the drug to one rat of each pair for 2 days, produced a thyroid gland[10] larger than that of the control in every instance. The

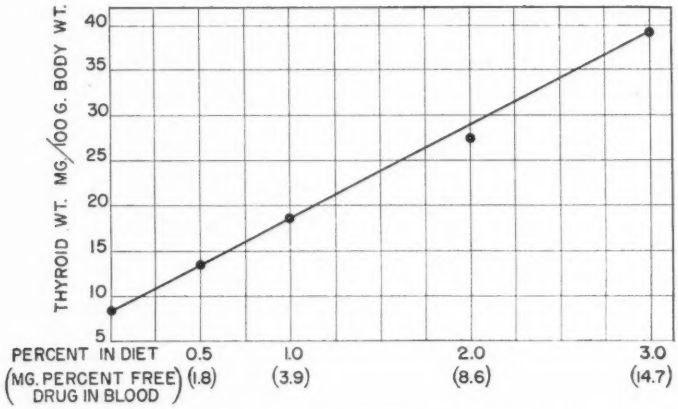

Fig. 1. Effect of feeding from 0 to 3 per cent of sulfaguanidine for 2 weeks on the thyroid weight (expressed as milligrams per 100 gm. body weight) of rats with an initial body weight of 40 to 50 gm.

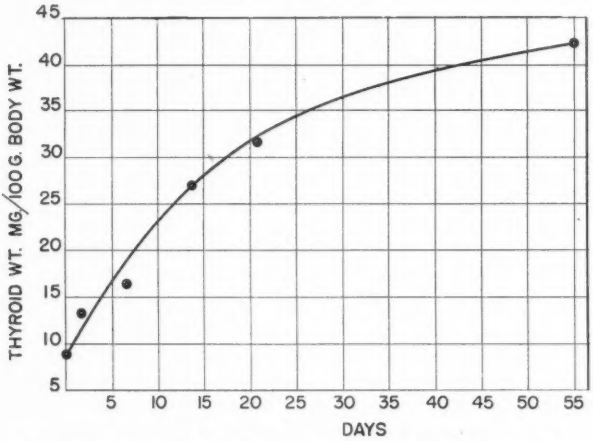

Fig. 2. Effect of feeding 2 per cent of sulfaguanidine for 2 to 55 days on the thyroid weight (expressed as mg. per 100 gm. body weight) of rats with an initial body weight of 40 to 50 gm.

average increase in thyroid weight calculated in this way was 34 per cent, and when tested for significance gave the high T value of 5.26. As is illustrated in figure 1, this rapid rate of increase in thyroid weight continued for the first 2 weeks on the diet, and thereafter began to diminish somewhat although it had not ceased at 8 weeks, when the increase equaled 465 per cent. The enlargement takes place in all directions

[10] Thyroid weight expressed as milligrams per 100 grams of body weight, unless otherwise noted.

and includes the isthmus, which becomes a wide, thick band. The gross appearance of the thyroid *in situ* of a young rat fed 2 per cent sulfaguanidine for 8 weeks is shown in figure 3. The thyroid of a control animal is shown for contrast (fig. 4). Microphotographs of the thyroid gland of a normal young rat are shown in figures 5 and 6.

Accompanying this increase in weight of the thyroids of immature rats receiving 2 per cent sulfaguanidine were histologic changes characterized by the continued heightening of the thyroid epithelium and its encroachment on the concomitantly diminishing and vacuolated colloid. The beginning of these changes was discernible at 2 to 4 days. By 7 days the epithelium had grown from the normal low cuboidal type of cell with its compact, elliptical, darkly staining nucleus, to high cuboidal and dis-

TABLE 1. EFFECT OF DIETARY SULFAGUANIDINE ON THYROID WEIGHT IN IMMATURE RATS[1]

Sulfaguanidine		No. of Rats	Days on Diet	Final Weight gm.	Thyroid Weight mg. per 100 gm.	Standard Deviation
% in diet	mg. % (free) in blood					
0	0	12	2	40–50	10.2±0.79	2.72
		13	7–14	70–80	8.8±0.45	1.61
		17	21	100–110	8.6±0.27	1.10
		20	50	150–180	7.9±0.31	1.38
0.5	1.75±0.31	23	14	70–100	13.9±0.31	1.48
1.0	3.88±0.28	13	14	90–100	19.0±1.04	3.76
		20	21	110–130	15.2±0.55	2.46
		11	50	150–180	19.8±0.68	2.27
		1	100	370	16.4	
2.0	8.57±0.17	12[2]	2	45	13.6±1.01	3.52
		8	7	60	16.3±1.04	2.96
		13	14	80	27.1±2.36	8.50
		6	21	100	31.7±3.63	8.90
		7	55	160	42.3±1.98	5.24
3.0	14.7±1.37	9	14	50–90	39.4±3.01	9.05

[1] Initial weight 35–60 gm.
[2] Each rat in this group was a litter-mate, matched for age and sex of one of the 12 animals in the first group of controls (Row 1).

tinctly columnar cells, with large spherical nuclei containing diffuse chromatin material (fig. 7). The colloid had diminished by approximately 50 per cent. After 10 to 14 days the epithelium was all of the columnar type and in many follicles it was so invaginated as to practically obliterate the lumen which in some instances contained shreds of palely staining colloid (fig. 8). Mitotic figures were frequently encountered.

After 14 days the papillary ingrowths, presumably giving rise to new follicles by budding, became more numerous and the gland resembled a solid tubular structure. Even at 60 to 100 days, however, the slit-like lumina still contained occasional shreds of watery colloid (fig. 9). Necrosis was not observed in this series of thyroids (fig. 10).

In immature rats receiving 1 per cent of sulfaguanidine the sequence of microscopic changes was the same, but the process did not progress so rapidly nor so far. At 14 to 21 days (fig. 11) the histology resembled that produced with 2 per cent of the drug in 7 days. At 50 days, the glands contained less colloid and the epithelium was predominantly columnar. This condition was slightly less severe than that produced by 2 per cent of the drug in 2 weeks.

Thyroid weights for immature rats receiving 1 per cent of the drug are included in table 1. Apparently the maximum size on this dietary level is reached in 2 weeks,

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although the histologic changes continue for some time thereafter. Clearly the thyroid weight, which has undergone no further increase by 50 days, does not necessarily reflect the degree of colloid depletion or epithelial elevation. The temporary decrease in thyroid weight from 19 mg. at 14 days to 15.2 mg. at 21 days would, according to Fisher's T test, come about by chance only one time in a hundred. However, before accepting this decline in weight as a consistent occurrence, it would be desirable to repeat the studies using paired litter-mates, in order to reduce the possible effect of genetic variability.

In view of the foregoing experiments, 2 weeks was selected as an appropriate period for testing the effect of different dietary levels of sulfaguanidine, namely, 0.5,

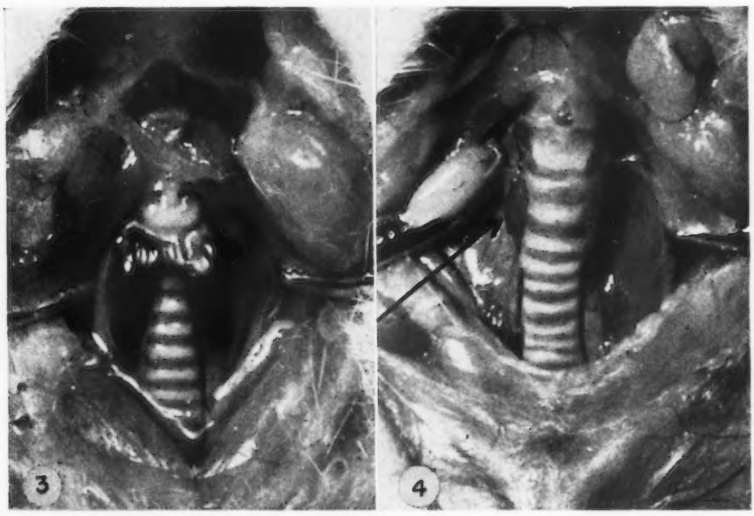
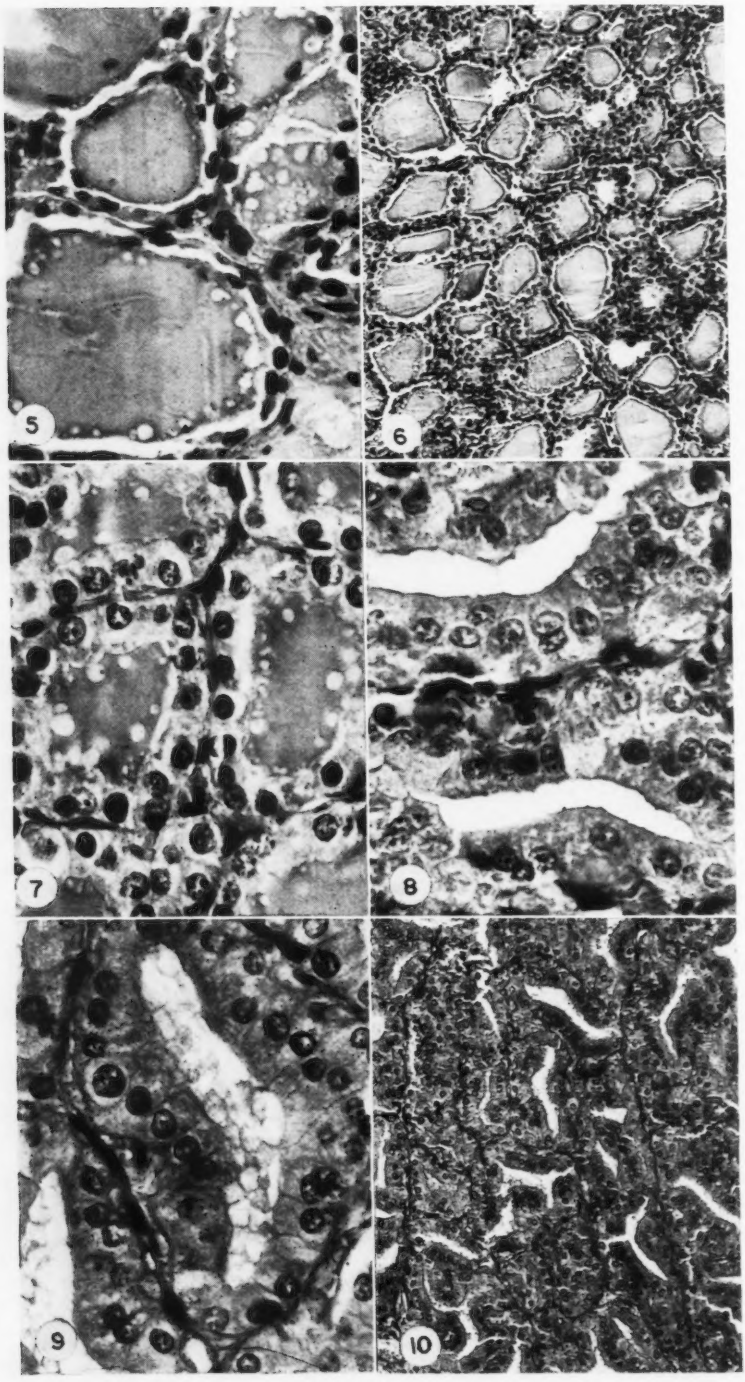


Fig. 3. THYROID OF YOUNG RAT FED 2 per cent sulfaguanidine for 56 days. Absolute weight of gland, 88 mg. Note hyperemia and the wide thick isthmus. Fig. 4. Litter-mate control. Absolute weight of gland, 15 mg.

1.0, 2.0, 3.0 and 4.0 per cent. At the 2 per cent dietary level the growth of the rats was excellent and concretions in the urinary system were seldom produced even after many months of treatment. On the 3 per cent dietary level about half of the animals died within 2 weeks. The remainder, however, grew fairly well during this period notwithstanding the fact that concretions were found in the urinary system in every case. Four per cent of the drug in the diet proved lethal for most of the young animals.

As is shown in figure 2 and table 1, the weight of the thyroid gland was directly proportional to the dietary level of sulfaguanidine over the ranges employed, and roughly equal to an increase over the normal of 10 mg. for each 1 per cent of the drug added. Five-tenths of a per cent (fig. 12) produced a 57 per cent increase in weight, an increase of high statistical significance as shown by Fisher's T test. The average blood level of free sulfaguanidine in this group amounted to only 1.75 ± 0.31 mg. per cent. Similar determinations made on the whole blood of rats receiving the other three dietary levels of sulfaguanidine revealed that the blood level of the drug was roughly proportional to the per cent in the diet. In these immature rats, therefore, increase in thyroid weight was closely correlated with the free circulating sulfaguanidine over a range of 1.75 to 14.7 mg. per cent (fig. 1).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In several instances the histologic appearance of the thyroid gland in immature rats fed 2 per cent of the drug for 30 days and then placed on the stock diet for 30 days, was examined. The follicles were filled with dense colloid and were lined with a low epithelium containing densely-stained elongated nuclei (fig. 13). It is probable that one revolution of a typical Marine (12) cycle had been produced. Whether or not intermittent administration of the drug will produce a large colloid goiter, and very prolonged administration necrosis, scarring and atrophy, is being determined.

At this point it seems pertinent to remark that no gross or microscopic changes in the thyroids of immature rats were produced during a 4-week period by feeding 1.5 per cent of sulfanilic acid or 0.55 per cent of guanidine carbonate either separately or in combination.

Growing adult rats. In these animals 1 and 2 per cent levels of sulfaguanidine fed over a period of 2 weeks produced histologic changes comparable to those observed in

TABLE 2. EFFECT OF SULFAGUANIDINE ON THYROID WEIGHT IN YOUNG ADULT MALE AND FEMALE RATS

Sulfaguanidine in Diet %	No. of Rats	Days	Initial Body Weight gm.	Final Body Weight gm.	Thyroid Weight mg. per 100 gm.
0	4			175	7.2
0	4			280	6.6
1.0	3	14	150	170–180	11.5
1.0	4	21	110	150–190	15.5
2.0	12	14	140	150–230	15.0
4.0	8	14	130	150–210	19.5

immature rats under the same conditions. However, the increase in thyroid weight as shown in table 2 was not so great. The feeding for 2 weeks of even 4 per cent of sulfaguanidine, a level tolerated by these animals, increased the thyroid weight no more than did 1 per cent of the drug in the immature rat. However, feeding the drug for longer periods of time to growing adult rats did produce striking thyroid enlargement. Two adult male rats, initially weighing 240 and 200 gm., were fed 2 per cent sulfaguanidine for 10 weeks. The drug level in the food was then increased to 4 per cent for an additional 8 weeks. When the animals were autopsied, the thyroid gland of the first animal weighed 288.8 mg., and that of the second, 195 mg., a very marked enlargement.

Old female rats. The effect of 1 and 2 per cent dietary levels of sulfaguanidine on thyroid weight in female rats one year of age or older is shown in table 3. A significant enlargement of the gland occurred only after 14 days. Histologically, however, the glands taken from 6 animals fed 2 per cent sulfaguanidine for 14 days showed definite signs of increased cellular activity. The colloid was vacuolated especially at the periphery. The epithelial cells were mostly of a high cuboidal type containing large vesicular nuclei. Mitotic figures were present and in some follicles a heaping up of the cells had begun. The difference in the rate of response of these old rats on 2 per cent sulfaguanidine as compared to younger animals was only in part associated with a lower blood level of free sulfaguanidine (caused by a lower food intake per gram of

Fig. 5. THYROID OF YOUNG RAT on stock diet. Low cuboidal epithelium and dense deeply-staining colloid are present (×600). Fig. 6. SAME AS FIGURE 5 (×150).

Fig. 7. THYROID OF YOUNG RAT fed 2 per cent sulfaguanidine for 7 days. Note rise in height of the epithelium, and beginning vacuolation of the colloid (×600). Fig. 8. THYROID OF YOUNG RAT fed 2 per cent sulfaguanidine for 14 days. Note the columnar epithelium and the almost complete absence of colloid. The large and spherical nuclei contain diffuse chromatin material (×600).

Fig. 9. THYROID OF YOUNG RAT fed 2 per cent sulfaguanidine for 102 days. Note that the epithelium is distinctly columnar, and that the colloid has not returned. Absolute thyroid weight 107 mg. (×600). Fig. 10. SAME AS FIGURE 9. Note the absence of scarring and necrosis (×150).

body weight and perhaps by differences in drug absorption and excretion) encountered in the former, namely, 3.5 per cent. After 2 weeks, approximately the same blood level in immature rats produced glands weighing 19 mg. and with far more advanced histologic changes. (Rats receiving 1 per cent of drug. See table 1.) As can be seen from tables 1, 2 and 3, the thyroid weights of our normal animals are in fairly close agreement with those reported by Remington, Remington and Welch (13) for animals of similar weight ranges. We made no effort to differentiate between the thyroid weights of normal male and female rats.

Activity of other sulfonamides. The positive effect of sulfonamides other than sulfaguanidine on the thyroid of the immature and young mature rat is shown in

TABLE 3. EFFECT OF SULFAGUANIDINE ON THYROID WEIGHT IN OLD FEMALE RATS

Sulfaguanidine in Diet %	No. of Rats	Days	Initial Body Weight gm.	Final Body Weight gm.	Thyroid Weight mg. per 100 gm.
0	8			250	8.0±0.54
1	2	14	230	235	8.0
1	2	21	260	265	11.0
1	2	28	275	300	11.4
2	13	14		263	9.2
2	4	21	250	240	11.0
2	4	28	220	230	15.2
2	4	35	230	230	15.6

table 4. The series of young mature rats fed 1 per cent sulfapyridine indicates that while the increase in thyroid weight may proceed at a slower pace than in the immature rat, it can and does reach the same level, 300 to 400 per cent.

It is quite clear from table 4 that sulfanilamide (fig. 14) is less effective than sulfapyridine, sulfadiazine and sulfaguanidine. It does not seem likely that this was due to a lower blood level of the former compound. On the other hand, the lower activity of dietary sulfathiazole may well be accounted for in this way. Blood determinations on young mature rats receiving 0.75 per cent of sulfadiazine and 1 per cent of sulfapyridine, gave average values for the free drugs of 13.2 ± 0.5 and 8.8 ± 1.1, respectively.

It is of interest that while sulfamethyldiazine produced a strong positive effect over a 2-week-test period this same compound without the para-amino group was without effect under the same conditions.

Activity of thioureas and other compounds. The tests carried out with thiourea itself on immature rats over a 2-week period disclosed a high degree of activity with respect to increase in thyroid weight. Apparently some effect was produced by the lowest dietary level of the drug employed, namely, 0.05 per cent, and the maximum effect was produced by 0.5 per cent (table 5). Increasing this to 2 per cent did not, during the 2-week period, raise the average thyroid weight past 37 mg. The subcutaneous administration of an aqueous solution of thiourea likewise produced marked thyroid enlargement in the rat. Histologically, the changes resembled those produced by the sulfonamides (fig. 15).

Recently, Kennedy (4), in attempting to isolate the goitrogenic agent in rape seed, reported that 20 mg. of allyl thiourea given daily by stomach tube to rats for an 8-week period caused marked thyroid hyperplasia. Larger doses, 50 mg. for a 10-day period, also produced enlargement, but not as marked as in the first instance. These findings are in agreement with ours.

Diethyl thiourea and allyl thiourea were active, while acetyl thiourea and thioacetamide (one NH_2 group replaced by $-CH_3$) were not. The latter was very toxic.

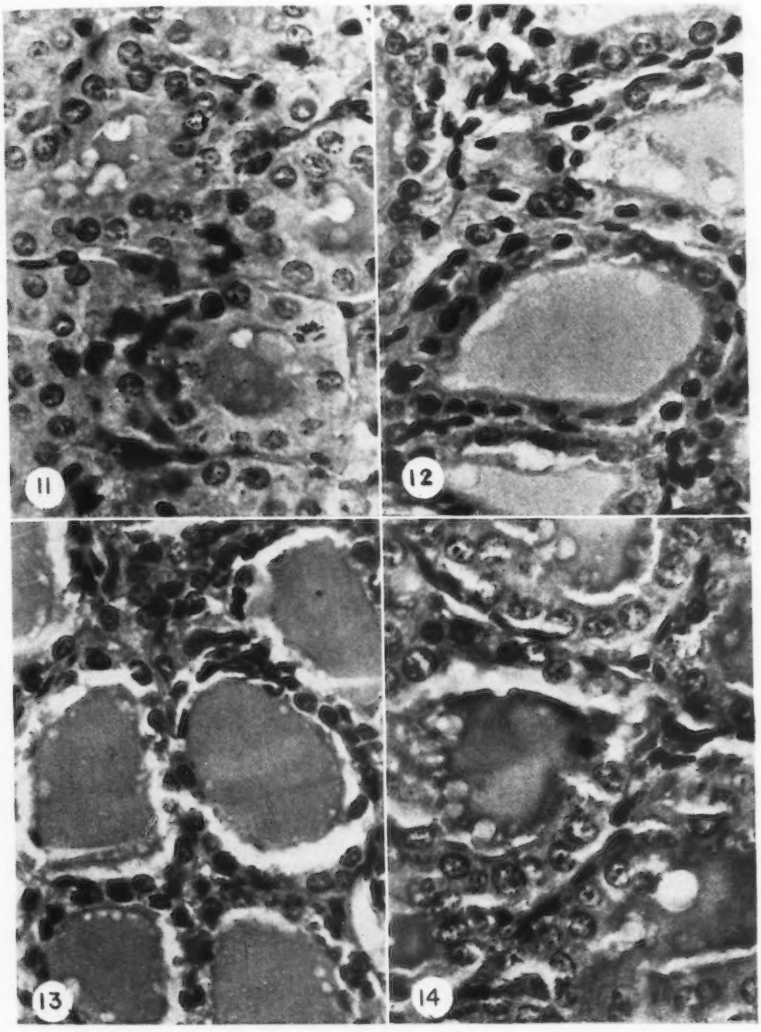


Fig. 11. THYROID OF YOUNG RAT fed 1 per cent sulfaguanidine for 14 days. Note the heightened epithelium and vacuolated colloid. See figure 19 for litter-mate control given 0.1 per cent NaI simultaneously with the drug (×600). Fig. 12. THYROID OF YOUNG RAT receiving 0.5 per cent sulfaguanidine for 14 days. Note the rise in the height of the epithelium and vacuolation of colloid (×600).

Fig. 13. THYROID OF YOUNG RAT fed 2 per cent sulfaguanidine for 14 days, and then placed on the stock diet for 30 days. The follicles are filled with dense colloid and lined with cuboidal epithelium (×600). Fig. 14. THYROID OF YOUNG RAT fed 1 per cent sulfanilamide for 25 days (×600).

The negative results obtained with phenyl thiourea were no doubt due to the low levels employed since Richter and Clisby (3) found that this compound produced thyroid enlargement. As has been mentioned previously, guanidine carbonate was negative. Urea gave similar results at a 5 per cent level. Negative results were obtained with sulfonal, cystine, cysteine, thiamine hydrochloride and the thiocyanates (table 5). Para-aminobenzoic acid produced slight but definite thyroid enlargement

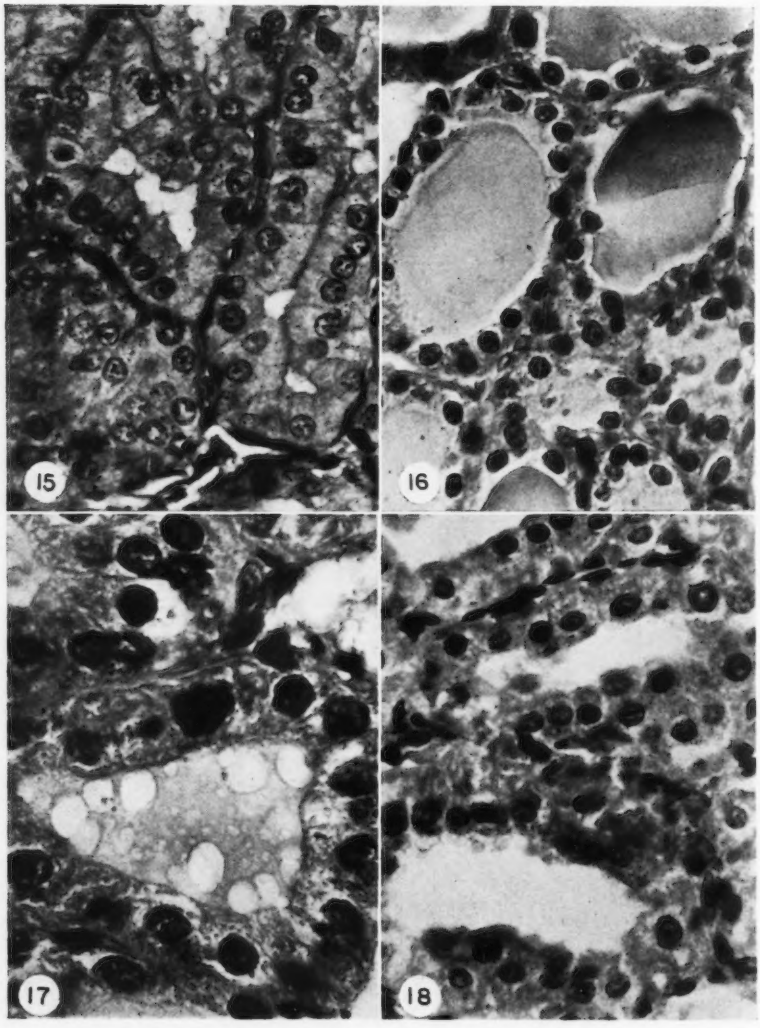


Fig. 15. THYROID OF YOUNG RAT fed 0.66 per cent thiourea for 21 days. Note that this figure is almost identical to figure 8 (2 per cent sulfaguanidine for 14 days (×600). Fig. 16. THYROID OF MOUSE (CF1 strain). Note the compact elliptical nuclei, dense colloid and cuboidal epithelium (×600).

Fig. 17. THYROID OF MOUSE (CF1 strain) fed 1 per cent sulfapyridine for 18 days. Note vacuolated colloid and high cuboidal cells (×1000). Fig. 18. THYROID OF MOUSE (Swiss strain) fed 3 per cent sulfaguanidine for 14 days. Note the higher epithelium and greater depletion of colloid than in figure 17 (×600).

but did not decrease the colloid or cause hypertrophy of the epithelium. This reaction is being investigated further.

The mouse. Thyroid enlargement was produced in the mouse by sulfonamides and thiourea, as is shown in table 6. The glands of CF_1 strain mice fed 2 per cent sulfaguanidine for 18 and 41 days showed increased cellular activity. The epithelial

cells had passed from the normal, somewhat high, cuboidal type, to a larger spherical type. The number of follicles was considerably increased, and colloid, particularly in the central ones, was vacuolated. Colloid was never reduced much below half of the normal, and transition of the epithelium to the high columnar cells such as was found in the rat did not occur (fig. 16, 17).

It is interesting that the thyroids of mice in the second group (Swiss strain) fed 1 per cent sulfapyridine for 14 days, showed a histologic reaction very similar to that observed in rats maintained on the same drug for a similar length of time. The epithelium was distinctly columnar and only remnants of shredded colloid were seen.

TABLE 4. THYROID ENLARGEMENT AND HISTOLOGIC CHANGES PRODUCED BY SULFONAMIDES IN IMMATURE AND YOUNG ADULT RATS

No. of Rats	Compound	Per Cent in Diet	Days	Initial Body Weight gm.	Final Body Weight gm.	Thyroid Data Weight mg./100 gm.	Thyroid Data Histology
5	Sulfanilamide	1.00	25	50	110		++
4	Sulfanilamide	1.00	58	35	125	14.0	++
4	Benzyl Sulfanilamide (Setazine)	1.50	30	90	170	9.8	—
2	Sulfathiazole	0.75 1.50	14 14 } 28	55	120	19.0	++
5	Sulfathiazole	1.50	14	150	165	12.5	+
3	Sulfasuxidine	1.50	28	60	145	9.0	—
3	Promin	2.50 1.25	5 9 } 14	75	90	16.0	+
2	Promin	1.25	22	70	100	17.0	+
4	Sulfapyridine	1.00	58	55	125	40.0	++++
4	Sulfapyridine	2.00 1.00	7 19 } 26	40	75	45.0	++++
1	Sulfapyridine	2.00	14	42	68	—	++++
2	Sulfapyridine	1.00	14	140	135	12.0	
2	Sulfapyridine	1.00	21	140	125	29.0	++++
2	Sulfapyridine	1.00	28	135	135	40.0	++++
2	Sulfapyridine	1.00	36	145	145	32.0	++++
4	Sulfadiazine	0.75	45	65	125	47.0	++++
6	Sulfadiazine	2.00 1.00	7 7 } 14	45	80	16.8	++++
3	Sulfadiazine	2.00 1.00	7 19 } 26	40	85	40.0	++++
6	Sulfadiazine	0.75	14	180	225	14.8	++
4	Sulfamethyldiazine-(para-amino group)	0.75	14	50	70	9.3	—
4	Sulfamethyldiazine	0.75	14	45	80	25.0	++++

++++ = total loss of colloid and columnar epithelium.
++ = approximately 50% colloid and high cuboidal and columnar epithelium.

Of the 6 mice given 3 per cent sulfaguanidine for 14 days, only 3 survived the test period. Histologic examination of these glands, likewise, revealed the same alteration, as described above (fig. 18). Since the first group of mice (CF1) were maintained on the drug diets for a longer period of time, a strict comparison of the results obtained in the two groups is not possible. We are repeating this experiment on the first strain of mice (CF1) using 14 days as the test period.

The dog. Two young dogs weighing approximately 4 kg. each were fed sulfaguanidine at a level of 2 gm. per kg. for 5 days and 5 gm. per kg. thereafter.

Histologic examination of the thyroids of these animals, killed after 14 and 21 days on the diet, revealed follicles lined with columnar epithelium and devoid of colloid. The thyroids from 2 control dogs receiving the same diet without the drug were normal. Unfortunately the thyroid weights for these 4 animals were not obtained.

TABLE 5. EFFECT OF THIOUREAS AND OTHER COMPOUNDS ON THE THYROID OF YOUNG RATS

No. of Rats	Compound	Per Cent of Drug	Days	Initial Body Weight gm.	Final Body Weight gm.	Thyroid Weight mg. per 100 gm.
22	Thiourea	0.05	14	50	75	13.0
5	Thiourea	0.25	14	80	105	29.2
9	Thiourea	0.50	14	60	85	37.4
7	Thiourea	1.00	14	45	65	37.4
7	Thiourea	2.00	14	45	60	36.2
6	Thiourea	110 mg. daily subq.	14	50	70	30.2
4	Acetyl thiourea	1.000	24	65	70	7.5
5	Diethyl thiourea	1.000	28	60	62	26.4
5	Phenyl thiourea	.015	24			
		.030	7 } 43	95	140	7.4
		.060	12			
3	Diphenyl thiourea	0.100	27			
		0.200	6 } 42	75	160	9.0
		0.300	9			
7	Allyl thiourea	0.500	14	60	55	16.4
5	Urea	5.000	30	40	140	9.4
2	Phenyl urea	2.000	30	130	180	8.0
6	Thioacetamide	0.250	3 } 14			
		0.125	11	75	50	8.1
4	Cystine	2.000	30	40	130	10.5
7	Cysteine	2.000	14	50	103	10.7
4	Thiamin	1.500	26	60	170	8.0
5	Sulfonal	0.500	14	65	105	11.0
5	Sulfonal	1.000	14	45	92	9.2
6	Para-aminobenzoic acid	3.0	31	45	95	14.4
4	Sodium thiocyanate	1.5	45	60	190	9.8
3	Ammonium thiocyanate	1.5	14	130	100	6.0
2	Ammonium thiocyanate	1.5	30 } 44	135	130	6.5
		3.0	14			

The guinea pig. We have been unable to produce histologic alterations or weight increase in the thyroid glands of guinea pigs fed 1 per cent sulfapyridine or 0.75 to 1.5 per cent sulfaguanidine. On a 1.5 per cent dietary level of the latter drug, free drug levels in the blood ranged around those produced in the immature rat with 0.5

TABLE 6. EFFECT OF SULFONAMIDES AND THIOUREA ON THE THYROID OF MICE

No. of Male Mice	Compound	Per Cent in Diet	Days	Initial Body Weight gm.	Final Body Weight gm.	Thyroid Weight mg. per 100 gm.	Histologic Change
3[1]	Control		18, 24, 41	16	26	10	—
3[1]	Sulfaguanidine	2	18	17	23	30	+[3]
5[1]	Sulfaguanidine	2	41	17	23	36	+
4[1]	Sulfathiazole	2	24	17	24	25	+
4[1]	Sulfapyridine	2	24	18	22	32	+
6[2]	Sulfapyridine	1	14	19	19	16	++[4]
2[2]	Sulfaguanidine	3	14	17	21	27	++[4]
6[2]	Sulfaguanidine	2	14	18	17	22	+
6[2]	Thiourea	1	14	21	18	17	+
6[2]	Control		14	18	19	8	—

[1] Carworth Farms, Inc., New City, Rockland County, N. Y. CF1 Strain, 6–7 weeks of age.
[2] Swiss strain, 6–7 weeks of age.
[3] + = Hyperemia and enlargement, heightened epithelium and vacuolated colloid.
[4] ++ = Hyperemia and enlargement, columnar epithelium and almost total loss of colloid.

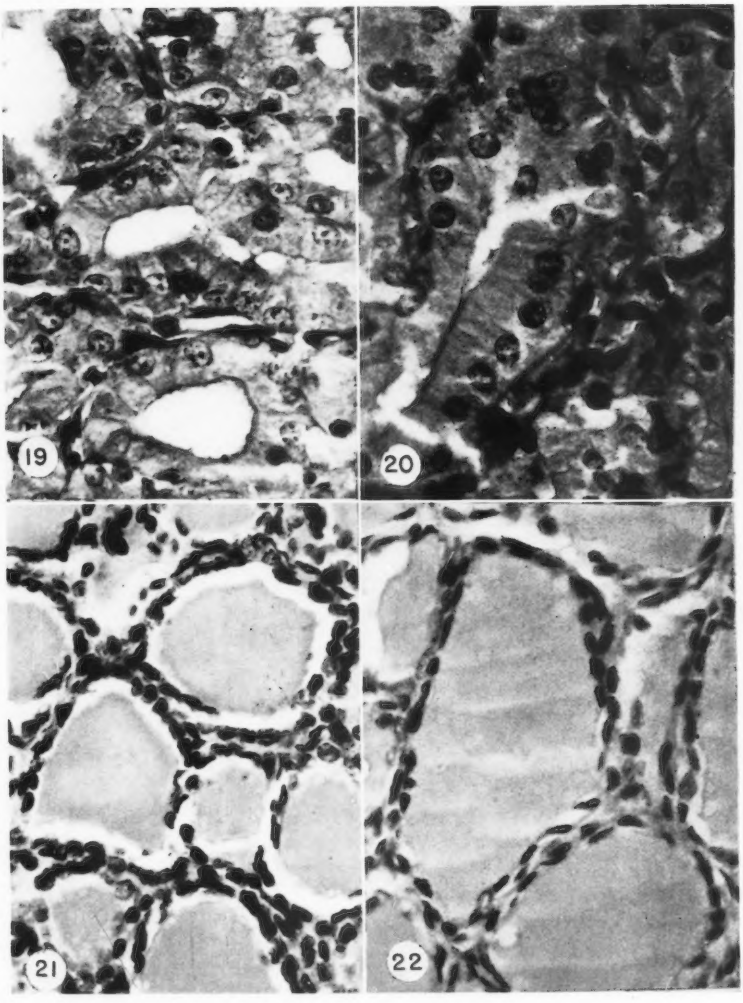
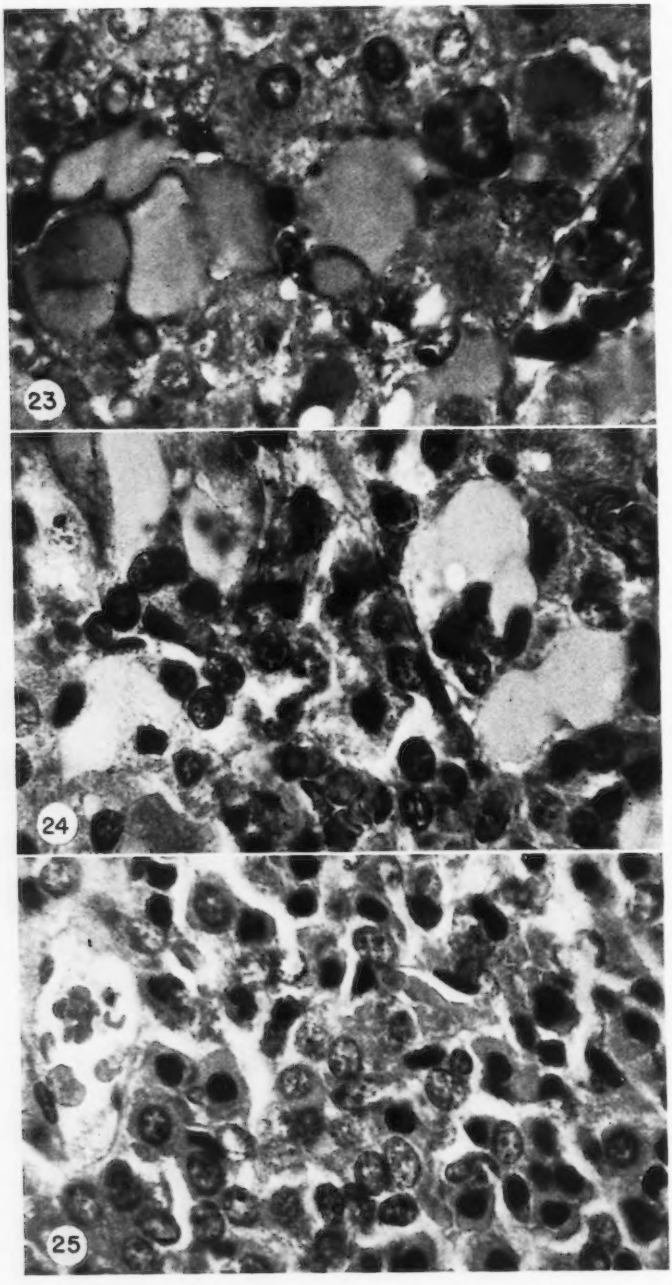


Fig. 19. THYROID OF YOUNG RAT fed 1 per cent sulfaguanidine simultaneously with 0.1 per cent of NaI for 14 days. (See litter-mate control, fig. 11.) Note the greater depletion of colloid (×600). Fig. 20. THYROID OF YOUNG RAT fed 4 per cent sulfaguanidine for 35 days (×600).

Fig. 21. LITTER-MATE CONTROL given 0.25 per cent desiccated thyroid simultaneously with 4% of the drug for 35 days. Note the normal appearance of the gland (×600). Fig. 22. THYROID OF YOUNG RAT fed 2 per cent sulfaguanidine for 14 days, and injected daily with thyroxin subcutaneously, 1 μg. per 10 gm. body weight (×600).

per cent sulfaguanidine, namely, 1.75 mg. per cent, and the possibility remains, therefore, that the results do not represent a true species difference but rather that the dietary level of sulfaguanidine employed was just insufficient to elicit a response. Higher dietary levels caused a lack of appetite, loss of weight and death.

The chicken. Ten 14-day-old chicks, weighing approximately 100 gm. each, were fed a commercial mash with 2 per cent sulfaguanidine for 14 and 30 days. The thyroid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glands were not enlarged over those of the controls, and histologic examination revealed well-filled acini lined with cuboidal epithelium. In the group receiving the 2 per cent dietary level, the free drug in the blood varied from 2.21 to 5.74 mg. per cent. In another group of 6 chicks fed 2 per cent sulfaguanidine for 2 weeks, increasing the level of the drug to 3 per cent for an additional 14 days was without effect, either grossly or histologically. In the group fed 2 per cent sulfaguanidine and later 3 per cent, the free drug levels in the blood varied from 7.9 to 14.6 mg. per cent. Acetylation of the drug also takes place in this species.

Older chickens, approximately 12 weeks of age and averaging 600 gm. in weight, fed 2 per cent sulfaguanidine for 14 and 21 days, likewise showed no appreciable increase in thyroid weight. Histologically, the glands were normal. The free drug levels in the blood in this group varied from 12.7 to 19 mg. per cent. In all drug-treated groups, the blood level necessary to produce thyroid alteration is higher than that which is required in the rat. The commercial mash used for the chickens plus 2 per cent drug was fed to a group of 6 young rats for a 2-week period to eliminate the possibility of a protective dietary factor in the mash. No such factor was present, since the rat thyroids were enlarged and hyperemic.

Under the conditions that we have employed, it is evident that the chicken is resistant to the action of sulfaguanidine with respect to thyroid alteration and that a true species difference exists. It seems pertinent to point out here that the chicken and rat are both susceptible to the goitrogenic agent present in the soybean, as shown by Sharpless, Pearsons and Prato (14) in the rat, and by Wilgus, Gassner, Patton and Gustavson in the chicken and rat (15).

Basal Metabolic Rate

While preliminary experiments on immature rats weighing from 50 to 100 gm. showed that sulfaguanidine definitely depressed the B.M.R., the behavior was too erratic to yield good quantitative results. Consequently, young 200-gm. adult rats were employed. After the normal basal metabolic rate for each individual had been ascertained by averaging the results of 3 weekly determinations, the animals were divided into two groups, one of which was fed 2 per cent sulfaguanidine.

The B.M.R. of the rats in each group was then determined at approximately weekly intervals and the percentage change from the previously established normal for each rat was calculated.

The average change for the control animals was subtracted from the average change for the drug-treated animals to determine the true effect of the drug on the metabolic rate. These results are given in table 7. When the first determinations were made, after 5 to 7 days of feeding the drug, the B.M.R. had already dropped 10 per cent. By 10 to 14 days it was −20 per cent, a level which was maintained to the end of the experiment at 40 days.

In the case of 2 rats, 449 and 450, the experiment was extended to 52 days, at which time the basal rates were −25 and −17 per cent, respectively. The drug was then omitted from the diet. Determinations made at 6, 24 and 37 days thereafter gave basal metabolic rates of −26, −21 and −24 per cent in the first instance, and −19, −13 and −21 per cent in the second. Fifty days after the last basal determina-

Fig. 23. ANTERIOR PITUITARY OF SEXUALLY MATURE RAT fed 2 per cent sulfaguanidine for 62 days. The acidophils are decreased in number, and vacuolated enlarged basophils are clearly seen (Mallory II ×1000). Fig. 24. ANTERIOR PITUITARY OF YOUNG RAT fed .75 per cent of sulfadiazine for 45 days. Note the nests of thyroidectomy cells (H & E ×1000).

Fig. 25. ANTERIOR PITUITARY of a young control rat. Note the abundance of acidophils (H & E ×1000).

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tion these animals were autopsied. The thyroids showed no hyperemia although they did appear to be slightly enlarged. Since they were fixed on the trachea no thyroid weights were taken. Histologic examination revealed follicles lined with very low epithelium surrounding an abundance of dense colloid.

The results on basal metabolism do not exclude the very important possibility that an abrupt transitory rise in oxygen consumption had occurred preceding the decline found when the first measurements were made at 5 days. In a few experiments on adult animals receiving 1 per cent thiourea a definite depression in the B.M.R. was also observed.

TABLE 7. DECREASE IN BASAL METABOLIC RATE PRODUCED BY THE ADDITION OF 2 PER CENT SULFAGUANIDINE TO THE DIET OF YOUNG ADULT MALE RATS

Treatment	Animal	Sex	Normal B.M.R.[1]	Percentage Change in B.M.R. at Various Days After Treatment						
				5–7	10–14	21–24	27–31	35–41	45	52
None	369	F	644	−3	+2	−5	−4	−4		
	417	F	602	+4						
	451	M	633	−2	−2	0	−2	−2		−1
	453	F	636	−6	−7	−6	−5	−5		
	468	M	730	−2	+1	0		−6		
	490	M	638	+7	−4	−3		−6		
Average				0	−2	−3		−5		
Sulfaguanidine	367	M	659		−19	−23	−24	−27		
	449	M	726	−10	−17	−20	−23	−32	−28	−25
	450	M	672	−1	−22	−16	−20	−17	−22	−17
	487	M	663		−20	−8	−10	−19		
	488	M	682	−12	−18	−26		−32		
	489	M	722	−15	−21	−26		−22		
	491	M	718	−9	−32	−25	−25	−27		
	492	M	659	−12	−25	−22	−19	−22		
Average				−10	−22	−21	−20	−25		
Difference				−10	−20	−18		−20		

[1] Average of 3 readings taken at weekly intervals. B.M.R. expressed as calories per square meter per 24 hours.

Prevention and Cure

A number of substances was tested for their possible protective action against sulfaguanidine by administering them simultaneously with the drug over a period of time and then weighing the thyroids of the treated animals. The substances employed fall into three groups, *a*) those chosen because of general nutritional considerations; *b*) sodium iodide; *c*) derivatives of the thyroid gland itself.

The results of experiments with the first group of substances are shown in table 8. There is no indication that either inositol, liver powder, fresh liver, para-aminobenzoic acid, cysteine, cystine, vitamin C or calcium pantothenate, prevented thyroid enlargement. The average thyroid weight of the treated animals is in every case above the maximal value found in normal untreated animals of comparable body weight. Quantitative comparisons between the effect of sulfaguanidine alone, and the effect of sulfaguanidine plus the different dietary factors shown in table 8, cannot readily be made in our material because of differences in duration of treatment, concentration of the drug, and age and weight distribution of the experimental animals, part of which are referable to the fact that many of the data are drawn from preliminary experiments made before certain features of our procedure had been standardized. Rough comparisons, however, suggest that at least two of the substances tested

(vitamin C) and para-amino-benzoic acid may actually enhance the thyroid effect of the sulfaguanidine, and this possibility is being investigated further.

In two experiments with iodine feeding (both in weight and in histologic appearance) the thyroids from animals treated with sulfaguanidine plus sodium iodide were compared with those of litter-mate sulfaguanidine-treated controls without iodine supplements in their diets.

TABLE 8. FAILURE OF DIETARY FACTORS TO PREVENT THYROID ENLARGEMENT BY SULFAGUANIDINE

No. of Rats	Sulfaguanidine %	Treatment	Days	Average Initial Body Weight gm.	Average Final Body Weight gm.	Thyroid mg. per 100 gm.	Range of Normal Thyroid Weights for Rats of Weight Ranges Indicated[1]	
							mg. per 100 gm.	gm.
6	2	Inositol 0.2 gm. %	8	40	55	19	10.2 ± 0.790 6.4 —13.60	40–50 (12)
2	1	Liver powder 10.0%	10	40	75	22	8.8 ± 0.45 7.1 —11.60	70–80 (13)
8	1	Para-aminobenzoic acid 0.1%	14	50	80	22		
6	2	Fresh liver 2 gm. daily	14	40	85	23		
6	2	Cysteine 2.0%	14	50	100	37	8.6 ± 0.27 7.0 —10.80	100–110 (17)
4	2	Para-aminobenzoic acid 2.0%	35	45	110	63		
6	2	Vitamin C 15 mg. daily	31	50	120	52		
4	2	Cystine, 2%	17	140	150	18	7.9 ± 0.31 6.00—11.00	150–180 (24)
6	1	Fresh liver 2 gm. daily	14	110	175	12	8.0 ± 0.54 5.0 — 9.80	175–280 (16)
4	2	Calcium pantothenate 0.2 gm.%	17	160	180	19		
2	2	Fresh liver 2 gm. daily	14	130	190	15		

[1] These are compiled from the data summarized from tables 1, 2, and 3 for control animals. Figures in parentheses indicate the number of animals on which ranges are based.

In the experiment recorded in table 9 the two groups of 7 rats each were both fed the McCollum stock diet plus 1 per cent sulfaguanidine for the periods indicated (14–20 days). The animals of *group B* were given in addition a dietary supplement of 0.1 per cent NaI, beginning 5 days before the sulfaguanidine treatment was instituted and continuing throughout the duration of the experiment. The average thyroid weight of the animals in the group treated with sulfaguanidine without extra iodine was 13.66 mg.; in the iodine-supplemented group, 16.49 mg. The difference is not statistically significant, and it clearly indicates that the iodine had no protective effect against the action of the sulfaguanidine. Histologic examination of the thyroids from the two groups confirmed this inference—there was actually a greater depletion of colloid in the iodide-supplemented group (fig. 19).

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TABLE 9. EFFECT OF SODIUM IODIDE ON THYROID WEIGHT OF YOUNG RATS FED 1% SULFAGUANIDINE

Sex	Duration of Treatment	Initial Body Weight gm.	Final Body Weight gm.	Thyroid Weight (absolute) mg.	Thyroid Weight mg. per 100 gm.
		A. Stock Diet and 1% Sulfaguanidine[1]			
M	14 days	55	129	15.6	12.1
F	14 days	51	108	14.4	13.3
M	14 days	58	137	26.2	19.1
F	14 days	59	124	12.8	10.3
M	20 days	60	165	19.6	11.9
F	20 days	51	123	18.2	14.8
F	20 days	45	118	16.6	14.1
Average		54.0	129.2	17.63	13.66
		B. Stock Diet + 1% Sulfaguanidine and 0.1% Na I[1]			
M	14 days	44	101	16.4	16.2
F	14 days	57	116	14.8	12.8
M	14 days	61	150	19.4	12.9
F	14 days	55	117	16.8	14.4
M	20 days	55	165	27.0	16.4
F	20 days	51	122	22.2	18.2
F	20 days	45	119	29.2	24.5
Average		52.6	127.1	20.83	16.49

[1] Each animal of *group B* is a litter-mate of the animal in the corresponding row of *group A*.

TABLE 10. EFFECT OF A PURIFIED DIET WITH AND WITHOUT NaI ON THE THYROID OF IMMATURE RATS FED 0.5% SULFAGUANIDINE

I. Purified Diet Only						II. Purified Diet + 0.05% NaI					
Litter No.	Sex	Initial Body Weight gm.	Final Body Weight gm.	Thyroid Weight mg.	Thyroid Weight mg. per 100 gm.	Litter No.	Sex	Initial Body Weight gm.	Final Body Weight gm.	Thyroid Weight mg.	Thyroid Weight mg. per 100 gm.
1	M	47	101	8.6	8.5	1	M	48	113	12.8	11.3
1	M	46	101	9.0	8.9	1	M	40	95	8.8	9.3
1	F	43	91	8.2	9.0	1	M	50	90	7.6	8.4
2	M	52	97	9.4	9.7	2	F	46	83	9.4	11.3
2	F	42	77	7.4	9.6						
3	F	42	79	8.4	10.6	3	F	47	91	7.8	8.6
3	F	46	83	10.2	12.3	3	M	46	94	9.4	10.0
3	M	48	105	10.8	10.3						
Average			91.8	9.0	9.86	Average			94.3	9.3	9.82
III. Purified Diet + 0.5% Sulfaguanidine						IV. Purified Diet + 0.5% Sulfaguanidine + 0.05% NaI					
1	M	48	107	11.4	10.6	1	F	45	91	14.8	16.3
1	M	46	106	12.0	11.3	1	M	46	97	12.6	13.0
1	M	45	104	13.8	13.3	1	M	47	109	15.0	13.8
2	F	48	90	11.8	13.1	2	M	57	116	18.0	15.5
2	F	46	85	10.2	12.0	2	F	40	81	14.0	17.3
3	M	50	100	13.6	13.6	3	F	44	81	15.2	18.8
3	F	44	86	10.4	12.1	3	F	47	82	15.2	18.5
3	F	38	71	12.0	16.9	3	F	42	67	14.0	20.9
Average			93.6	11.90	12.86	Average			90.5	14.85	16.76

In the next experiment (table 10) the basal ration used for both iodide-supplemented and control groups was a purified diet[11] containing 367.5 μg. of iodine per kg.[12] In order to reduce the iodine intake to a minimum, the mothers of the 3 litters of rats used in the experiment were deprived of the usualy weekly iodine water and placed on the purified diet, in one case 4 days after, and in the other two cases 11 days after, the birth of their young. Each female received an oral supplement of 20 mg. of alpha-tocopherol weekly, and the young rats were each given 5 mg. of alpha-tocopherol at 12 days of age and at weekly intervals, thereafter.

At 21 days of age the young rats from the 3 litters were divided into 4 groups. *Group I* was continued on the purified diet. *Group II* received this diet plus .05 per cent NaI. *Groups III* and *IV* were given 0.5 per cent sulfaguanidine, and the latter group received also a dietary supplement of 0.05 per cent NaI. In all cases the treatment was continued for 14 days.

There was no appreciable difference in thyroid weight between groups *I* and *II* (table 10), and the histologic appearance of the thyroids was entirely normal in these two groups. The acini were filled with an abundance of colloid and were lined with cuboidal epithelium. It is clearly evident that the purified diet is not goitrogenic in itself. According to Levine et al. (16), 1 to 2 μg. of iodine per day as iodide is adequate to meet the needs of the rat for this element. An animal eating between 7 and 10 gm. of the purified diet per day would obtain between 2.6 and 3.7 μg. of iodine daily, an amount more than adequate to satisfy his needs. In both of the sulfaguanidine-treated groups (*III* and *IV*), there was a definite increase in thyroid weight, and histologic examination revealed unmistakable hyperplasia. Finally, among the sulfaguanidine-treated groups, the average thyroid weight was higher in the iodide-supplemented animals (14.85 mg. or 16.76 mg. per 100 gm. body weight) than in the group on the purified diet (11.90 mg. or 12.86 mg. per 100 gm. body weight). The difference is statistically significant. Moreover, histologic examination showed a more marked cellular reaction in the thyroids of *group IV* (iodine-supplemented) than in those of *group III*. The follicles contained much less colloid, and the epithelium was predominantly columnar, while in *group III* about 50 per cent of the colloid still remained, and the epithelium was predominantly high cuboidal. More experiments with iodine feeding are in progress, but the evidence already presented strongly indicates that iodide-feeding of animals treated with sulfaguanidine on low iodine diets, far from exerting any protective action, actually results in an exaggerated response of the thyroid to the drug.

In order to eliminate the unlikely possibility that the drugs prevented iodine absorption, a group of rats fed stock diet plus 1 per cent sulfaguanidine was injected subcutaneously with 100 μg. of iodine daily (as a NaI solution) (table 11). This level far exceeds the amount required to prevent goiter in rats on the low-iodine diet of Levine, Remington and von Koltnitz (16) or on the raw soybean diet of Sharpless et al. (14). Our attempts at parenteral treatment met with complete failure.

The administration of large doses of iodine to rats with enlarged thyroids had no harmful effects. The feeding of 0.25 per cent NaI to young and growing adult rats, on the stock diet along with 1 and 2 per cent of the drug was also without effect on the thyroid enlargement or histologic reaction (table 11).

In contrast with the foregoing results were those obtained with desiccated thyroid and thyroxin (table 12). With either substance the thyroid enlargement and

[11] Dextrose 56.0; salts #51 6.0; yeast 10.0; casein (acid-washed) 20.0; lard 8.0; haliver oil fortified with viosterol 26 drops per kilogram of diet.

[12] We are indebted to Dr. William C. Foster of the Hahnemann Medical College, Philadelphia, Pa., for the determination of the iodine content of the purified diet used in our experiment.

TABLE 11. EFFECT OF PARENTERAL AND ORAL ADMINISTRATION OF SODIUM IODIDE ON THE THYROID WEIGHT OF RATS FED 1 AND 2% SULFAGUANIDINE

No. of Rats	Sulfaguanidine %	Treatment	Days	Average Initial Body Weight gm.	Average Final Body Weight gm.	Thyroid mg. per 100 gm.
8	1	Sodium iodide 0.12 mg. daily subcut.	14	50	90	19
6	2	Sodium iodide 0.25%	14	164	190	15
4	1	Sodium iodide 0.25%	14	170	225	12
2	0	Sodium iodide 0.25%	14	176	226	8

histologic change produced in immature rats by 2 or 4 per cent of sulfaguanidine was entirely prevented (fig. 20, 21). The daily doses of thyroxin were administered subcutaneously at levels of 100, 10 and 1 μg. per 10 gm. of body weight. At the last two levels, litter-mate controls received sulfaguanidine alone. Even the lowest dosage of thyroxin not only prevented the thyroid enlargement but reduced the weight of the gland below the normal for animals of this size (see table 1). Histologically, the thyroid glands of the treated animals possessed a low cuboidal epithelium and an abundance of colloid (fig. 22).

TABLE 12. PREVENTION OF THYROID ENLARGEMENT BY DESICCATED THYROID AND THYROXIN

No. of Rats	Sulfaguanidine %	Treatment	Days	Initial Body Weight gm.	Final Body Weight gm.	Thyroid mg. per 100 gm.
3	2	Desiccated Thyroid, 0.25%	7	40	52	10.8
3	2	Desiccated Thyroid, 0.25%	21	35	100	8.2
3	2	Thyroxin, 100 μg. per 10 gm. Body Weight daily, subcut.	7	40	65	7.8
3	2	Thyroxin, 100 μg. per 10 gm. Body Weight daily, subcut.	14	40	95	6.3
4[1]	2	Thyroxin, 100 μg. per 10 gm. Body Weight daily, subcut.	21	40	110	6.0
4[1]	2	No Treatment	14	50	110	29.5
4[2]	2	Thyroxin, 10 μg. per 10 gm. Body Weight, daily subcut.	14	55	105	6.2
4[2]	2	No Treatment	14	40	75	35.5
5	2	Thyroxin, 1 μg. per 10 gm. Body Weight, daily subcut.	14	40	80	6.2

[1] Litter mates.
[2] Litter mates.

The daily administration of 10 μg. of thyroxin per 10 gm. of body weight to rats receiving 2 per cent of sulfaguanidine, and with basals of −20 per cent, brought about a rise in oxygen consumption within 24 hours, which resulted in basals of +20 per cent by the eighth day.

Changes in the Anterior Pituitary of the Rat

A number of investigators (4, 6, 17–20) have shown that changes in the cellular pattern of the anterior pituitary are associated with dysfunction of the thyroid. Marine, Rosen and Spark (19) found not only cellular changes in the anterior pituitary of rabbits with parenchymatous goiter, but also an increase in weight of the gland. These workers found that the administration of iodine or desiccated thyroid to these

animals restored the cells to normal. Recently, Kennedy (4) reported that the oral administration of allyl thiourea to rats produces not only thyroid hyperplasia but an alteration of the cellular pattern of the anterior pituitary as well. These pituitary changes are similar to those found in animals fed rape seed, as fully reported by Griesbach (6). Beginning pituitary changes can be detected as early as 7 days in young rats receiving 45 per cent rape seed in their diets (6).

Since thyroid hypertrophy and hyperplasia occur in animals fed 2 per cent sulfaguanidine with such rapidity, our attention was early directed to the pituitary. The use of hematoxylin and eosin for staining purposes was not satisfactory, and differential stains for both the acidophils and basophils were used. By means of this latter technique, it was possible to detect a degranulation and decrease in number of the acidophils, vacuolation and increase in number and size of the basophils as early as the 14th day. These changes correspond closely to those reported following thyroidectomy (20). Animals maintained on the 2 per cent sulfaguanidine diet for longer periods show more marked changes, i.e., almost complete absence of acidophils, and a progressive increase in the number of so-called thyroidectomy cells (fig. 23). Similar changes in the pituitary were found in animals fed other sulfonamides and thioureas (fig. 24, 25).

Detailed studies of the changes in the pituitary, and pituitary weights of animals fed sulfonamides and thioureas will be reported in a subsequent publication. The effect of iodide and desiccated thyroid on the pituitary is also being studied.

The testes of several male rats after 60 and 100 days on the 2 per cent sulfaguanidine diet were found to be normal on histologic examination.

The ovaries of a number of females were likewise examined and no evidence of alteration was detected. The estrous cycles of 4 young females given 2 per cent sulfaguanidine for 4 weeks were observed for a 3-week period. The estrous cycles were normal. One of these females was impregnated by a stock male and gave birth to a litter of living young.

Mechanism of Action

In a consideration of how the thyroid effect of the sulfonamides is produced, the first question relates to the site of action. It might be supposed that the drugs act within the alimentary tract, by inactivating some dietary constituent or by inhibiting the formation of some essential metabolite which the intestinal flora normally produce. The inactivity of a poorly absorbed sulfonamide, succinyl sulfathiazole, weighs against this hypothesis, however; and the effectiveness of parenteral administration of thiourea seems to eliminate it completely. Alternatively, the drugs might act after absorption, and we are left to infer that this is the case.

What then is the action of these drugs within the body? It can hardly be accounted for by reference to vague toxic or narcotic effects, for guanidine carbonate and thioacetamide, both toxic substances, failed to enlarge the thyroid; and sulfonal, a hypnotic, was without effect in concentration as high as 1 per cent. Furthermore, sulfaguanidine caused considerable thyroid enlargement at dietary concentrations which neither restricted growth nor produced detectable calculi in the urinary system.

It likewise seems improbable that the thyroid-metabolic effect is secondary to anemia or cyanosis. Richardson's (21) excellent study on mice has shown that sulfanilamide is much more effective than sulfaguanidine in producing anemia, sulfhemoglobinemia and reduced oxygen-combining capacity of the blood, but our data give no indication that there is a parallel difference between the effects of the two drugs on the thyroid (tables 1 and 4).

Hemoglobin determinations in our own sulfaguanidine-treated animals have re-

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vealed no depression of the hemoglobin level. Ten young rats fed 1 or 2 per cent of sulfaguanidine for 2 weeks showed hemoglobin values of 13.2 and 13.5 gm., respectively, as compared with an average of 13.4 gm. for the control animals. The remaining possibilities would seem to be that the effect of the drugs is achieved through one or several of the following mechanisms: *a*) Retention of thyroxin by the thyroid gland. *b*) Elevation of tissue requirements for thyroxin (e.g., through the inhibition of some enzyme system). *c*) Destruction or inactivation of the circulating hormone. *d*) Depression of the rate of thyroxin formation.

The first of these possible mechanisms is rendered unlikely by the fact that there is a reduction in the organic iodine content of the colloid in rats treated with sulfaguanidine which is detectable as early as the second day of treatment, as shown by Gersh (22); and, by our own observation that the colloid itself disappears almost completely soon afterward, from the 10th to the 14th day.

Both the second and third mechanisms suggested above imply the existence of functional hyperthyroidism with metabolic hypothyroidism, while the fourth involves true functional hypothyroidism, as well as metabolic hypothyroidism. This histologic picture of the thyroid in our drug-treated animals does not permit a decision as to which of these two general situations exists, unless it is assumed that hyperplasia necessarily means increased hormone production. This assumption, we believe, is not justified. Collip and Anderson (23), for example, have produced thyroid hyperplasia without a rise in the B.M.R. by administering appropriately balanced quantities of thyrotropic and antithyrotropic hormones to hypophysectomized rats. In our experiments the hyperplasia probably is mediated through the pituitary.

Because of the inadequacy of morphologic criteria for distinguishing between functional hyperthyroidism and functional hypothyroidism, and in the absence of adequare chemical methods for the determination of thyroxin in biological systems, the following experiments were designed in the hope of securing some indirect evidence on the nature of the mechanism.

Effect of Sulfaguanidine on the Basal Metabolic Rate of Thyroidectomized Rats

Five young actively-growing female rats weighing between 165 and 200 gm. were thyroidectomized. These animals were between 100 and 145 days of age at the time of operation. Following the operation, a 4-week interval was allowed to elapse before B.M.R. determinations were made at weekly intervals. Four such determinations were made on such animals, and the average rate computed before 2 per cent of sulfaguanidine was fed for 2 weeks. The results are shown in table 13. It will be noted that in two rats (4 and 5) the persistence of a high metabolic rate following the operation on the thyroid suggests that the thyroidectomy was incomplete, and in these animals there was the usual drop in B.M.R. following administration of the drug. In the remaining 3 animals, however, in which the low B.M.R. after operation is indicative of successful extirpation of the gland, no significant drop was produced by sulfaguanidine. These preliminary results indicate that the drug has no peripheral effect in the absence of the thyroid hormone, or at least no effect comparable to that produced in unoperated animals (table 4). The implication is that an increase in tissue requirements (mechanism *b*) does not occur in response to the drug. Next, thyroxin at a dosage level of 0.7 μg. per 10 gm. of body weight, injected subcutaneously for 3 days, was administered to the thyroidectomized animals under continued treatment with sulfaguanidine. It will be seen from table 13 that there was an increase in the B.M.R. of 4 animals of 24.0 to 29.3 per cent, as measured on the 5th day. One animal (2) gave an abnormally high response of 63 per cent. This may be compared with the elevation in B.M.R. reported by Meyer and Wertz (24) as the result of

administering thyroxin to thyroidectomized rats tested under conditions similar to ours, except that the thyroxin was given orally. In their animals 0.75 μg. per 10 gm. of body weight gave an average increase in the B.M.R. of 28.8 per cent. It would appear from the results of our experiment that sulfaguanidine does not destroy or inactivate the circulating thyroxin to any great extent. The results also tend to support the inference from the preceding experiment that the drug does not act by increasing tissue requirements. For, if either of these mechanisms (*b* or *c*) were involved, we should expect a diminished response to thyroxin in thyroidectomized animals receiving sulfaguanidine, as compared with the response found by Meyer and Wertz (24) in untreated thyroidectomized animals. However, in view of our ignorance of the thyroxin requirements of normal animals, and of the action of the hormone generally, these experiments can hardly be regarded as conclusive; in any case, additional quantitative data are desirable, and work in this direction is being continued. (Experiments with 6 additional rats have given similar results).

TABLE 13. EFFECT OF 2 PER CENT SULFAGUANIDINE ON THE B.M.R. OF THYROIDECTOMIZED RATS AND THEIR RESPONSE TO THYROXIN

Rat	Average B.M.R.[1]	Percentage Change from Average B.M.R. Produced by Sulfaguanidine		Percentage Increase Over Last B.M.R. Produced by Thyroxin[2]	Actual B.M.R. Produced by Thyroxin[2]
		7 days	14 days		
1	451	+ 4.7	+ 6.0	+28.2	613
2	441	− 4.1	+ 1.0	+63.2	726
3	515		− 7.2	+25.0	637
4	592	− 8.4	−11.8	+24.0	647
5	632	−16.6	−30.0	+29.3	574

[1] Average of 4 weekly determinations, the first of which was made approximately 1 month after operation. Expressed as calories per square meter per 24 hours. For B.M.R. of normal rats of same size and age see Table 7.

[2] 0.7 μg. per 10 gm. of body weight subcutaneously for 3 days. B.M.R. determined on fifth day.

We believe that the evidence, as far as it goes, from our own experiments and from those of others reported in this section, tends to exclude as likely mechanisms through which the thyroid-metabolic effect is produced *a*) retention of thyroxin by the thyroid gland, *b*) elevation of tissue requirements and *c*) destruction or inactivation of the circulating hormone. This would leave the depresssion of the rate of thyroid function, as the most probable mechanism.

In tracing further the chain of processes involved in the thyroid-metabolic effect we must take into consideration the possible intermediation of the pituitary. A study of this aspect of the problem, in coöperation with Gersh and his colleagues (25), has in fact established that the presence of the hypophysis is essential for the production of thyroid hyperplasia and hypertrophy by sulfaguanidine. The average (absolute) weight of thyroids from the 10 mature hypophysectomized rats fed 2 per cent of the drug daily for 14 days was 3.85 mg. as compared with an average of 5.25 mg. for 8 operated animals not receiving the drug; that is, treatment with sulfaguanidine failed to enlarge the thyroid in animals which lacked the hypophysis. Histologically, the thyroid glands of the hypophysectomized animals treated with sulfaguanidine were indistinguishable from those of the untreated controls, revealing an abundance of deeply-staining, dense colloid, low cuboidal epithelium and elliptical nuclei. In no case was there any sign of hyperplasia.

According to Marine (26), thyroid hyperplasia is due to a deficiency of iodine, and this deficiency may be a relative or absolute one. Even though the iodine intake

is high it is utilized with such rapidity that the iodine content of the gland itself falls, and hyperplasia results. A good case in point of a relative iodine deficiency is the goitrogenic effect of cabbage in rabbits, which is prevented completely by iodine (27). The goitrous agent present in cabbage is thought to inhibit tissue oxidation.

Since the administration of iodine in minute amounts is protective against goiter produced by cabbage in rabbits (27) and by soybean in rats and chickens (14, 15), our attention was early directed toward an alleviation by iodine of the thyroid metabolic effect produced in rats by the sulfonamides and thioureas. The administration of iodine in small or large quantities has conferred no protective action on the thyroids of our animals fed 0.5, 1 and 2 per cent sulfaguanidine. Actually, iodine in two experiments (0.5 and 1 per cent sulfaguanidine) aggravated the situation with reference to both the enlargement and histologic changes. At present we have no explanation for the apparent exaggeration of the sulfaguanidine effect produced by increasing iodine intake. In any case, it does seem to indicate that the mechanism involved in thyroid alteration produced by the sulfonamides and thioureas is not identical with that of previously described goitrogenic agents.

On the basis of the present evidence, our conclusion is that the sulfonamides and thioureas probably exert a depressing influence primarily on the functional activity of the thyroid, and hence on the B.M.R. and that the thyroid hyperplasia is a reflection of increased pituitary activity resulting from this depression.

SUMMARY AND CONCLUSIONS

Hyperemia and enlargement of the thyroid gland has been produced in rats, mice and dogs by the administration of sulfonamides and thioureas. Histologically, there is a reduction in colloid and an increase in the height of the thyroid epithelium. Thyroidectomy cells are found in the pituitary. The reaction does not occur in the chick or the guinea pig. In immature rats receiving from 0.5 to 3.0 per cent of dietary sulfaguanidine (free sulfaguanidine in the blood, 1.75 to 14.6 mg. per cent) the thyroid weight at the end of 2 weeks was proportional to the dietary level (roughly proportional to the blood level) and represented an increase in weight of 60 to 350 per cent. Two per cent sulfaguanidine (free sulfaguanidine in the blood 8.6 mg. per cent) produces in 2 days an increase in thyroid weight of 35 per cent. The increase in size using this dietary level of the drug was, during 55 days, a logarithmic function of the time. Sulfanilamide was less active than sulfaguanidine, sulfadiazine or sulfapyridine in producing thyroid enlargement. Thiourea was approximately 8 times more effective than the same dietary level of sulfaguanidine during a 2-week-test period. Diethylthiourea and allylthiourea were also active while acetylthiourea and thioacetamide were negative under the same conditions. The rate of thyroid weight increase in rats fed a given level of sulfaguanidine decreases with age. The rate of histologic response decreases to a lesser degree. This age difference can only partially be accounted for by a decrease in the blood level of the drug. In sexually mature male rats fed 2 per cent of sulfaguanidine the B.M.R. is −10 at 5 to 7 days and −20 at 10 to 14 days. The thyroid-metabolic effect produced in rats on a stock diet is not prevented by a number of dietary factors including para-aminobenzoic acid, vitamin C and liver. It is not prevented by iodine. This effect is entirely prevented by thyroxin at a dosage level of 1 μg. per 10 gm. of body weight. Hypophysectomy likewise prevents the thyroid alteration resulting from feeding 2 per cent sulfaguanidine to rats. Sulfaguanidine does not further depress the B.M.R. of thyroidectomized rats nor prevent their response to small doses of thyroxin. The thyroid enlarging effect of the sulfonamides and thioureas is probably mediated through the anterior pituitary. That other causative factors may be involved in this effect is not excluded.

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ACTION OF CERTAIN SULFONAMIDES AND OF THIOUREA UPON THE FUNCTION OF THE THYROID GLAND OF THE RAT[1]

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Goiterous changes in the thyroid gland have been observed to follow a variety of experimental conditions. A century ago iodine was used in the treatment of thyroid enlargement and it has become well established that hyperplastic goiters ensue when the dietary iodine is reduced below a certain level. Although iodine deficiency is said to be the commonest cause of thyroid hyperplasia in the human being and in experimental animals, a great many agents have been seen to cause a similar thyroid effect in such animals as rodents, ungulates, birds, fish and man. Reference to the extensive literature on this subject shows that the following agents have, under the conditions of the several experiments, induced thyroid enlargement: high protein diets (1–5), diets containing a large proportion of liver (4–7), fat (5), soybeans (8), or calcium salts (9–11), polluted water, bacterial cultures, fecal filtrates and filtrates from cultures of anerobic bacteria (12), diets composed largely of cabbage or related greens (13, 14), the feeding of the seeds of several species of Brassicae (15) and the administration of various nitriles, especially methylcyanide (16), arsenic salts (17) and thiocyanates (18–20).

Several detailed reports (8, 13, 14, 15, 16) have shown that the thyroid enlargement induced by certain of the above-mentioned substances is completely inhibited by increasing the daily intake of iodine, and it is a current opinion that these goiterigenic agents exert their effect upon the thyroid by influencing iodine metabolism. The substances in question are believed to raise the iodine requirement so that, when given in conjunction with diets which are low in this element, a true iodine-deficiency goiter is induced. When iodine supplements are given the thyroid hyperplasia is inhibited.

Interest in the field of goiterigenic agents was recently reawakened by the striking observations of Mackenzie, Mackenzie and MacCollum (21) and of Richter and Clisby (22) on agents which induce marked thyroid hyperplasia in rats even though adequate or excessive iodine is supplied. The former workers observed that sulfaguanidine when fed to rats as 1 to 2 per cent of the food induced a 3 to 8-fold increase in thyroid weight within 16 weeks. Similar enlargement, marked cellular hyperplasia and loss of colloid were noted by Richter in rats given phenylthiourea by injection or in the drinking water. The action of neither of these substances was inhibited by the administration of relatively large doses of iodine. Mackenzie and Mackenzie (23) have shown that several of the sulfonamides exert a goiterigenic effect on rats, but that neither guanidine nor sulfanilic acid are effective.

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It is the purpose of the present investigation to examine the mechanism of the thyroid hyperplasia that follows the administration to rats of substances of these types.

METHODS

Most of the experiments dealing with the mechanism of the goiterigenic effect were conducted using sulfaguanidine. Many of these were later repeated with thiourea, and certain ones with other related compounds. The present paper is concerned with the goiterigenic action of sulfaguanidine, sulfathiozole, sulfapyridine, sulfadiazine, sulfanilylurea, sulfasuxidine, sulfanilamide and thiourea.

These substances were administered to rats of the Long-Evans strain by subcutaneous injection, by stomach tube or by incorporation in the diet or the drinking water. The diet was Purina fox chow, with no supplements of greens or vitamins. The food was administered in the form of a powder from specially constructed feeding cups. The chemical compound when admixed with this powder formed a homogeneous material from which selection was impossible.

The majority of the animals used were 3 to 4 weeks old at the beginning of the experiments and most of these were female. Newborn and adult animals of both sexes, and pregnant females were also used. At the end of the experiment the animals were killed with illuminating gas, and the thyroid together with the adjoining trachea and lower portion of the larynx were removed *en bloc* and fixed in 5 or 10 per cent formalin. Sections were stained with hematoxylin and eosin or with eosin and methylene blue. Operative procedures were done under ether anesthesia by the commonly used technics. Oxygen consumption was measured by a simplified closed circuit apparatus in a room the temperature of which was maintained between 25 and 29° C. The animal was placed in a ½ or 1-pint preserve jar, and the temperature of the air within this jar was maintained manually at 27 to 29° C. by adjusting the temperature of an external water bath or by means of an adjustably placed electric bulb. This flexible but crude temperature control was necessitated by the markedly hyperthyroid or hypothyroid states of certain animals which would frequently die of excessive heating or chilling, respectively, when the vessel was maintained at a constant external temperature of 28° C. The rate of oxygen consumption was read from a small spirometer which recorded on a constant speed kymograph. For each determination records were taken for periods of 30 to 60 minutes and the calculations were made from those portions of the tracings which, from their regularity, indicated a minimal activity of the animal. As an index of the total energy metabolism, measurements were made of the daily food intake. The animals were weighed each day, and the rate of growth, together with the food intake, provided a rough measure of the total metabolic rate. Determinations on the level of sulfonamide in the blood were made by the method of Bratton and Marshall (24) on 0.02 or 0.1 cc. of blood obtained from the tip of the tail.

RESULTS

Effect on growth. When fed to normal young rats, sulfaguanidine was well tolerated at concentrations of 0.5 to 2.0 per cent by weight of the food. The animals ate this ration normally and gained in weight at a rate equal to that of the controls for the first few weeks. Figure 1 shows the growth rate and food intake of a group of 12 rats fed the diet containing 2 per cent sulfaguanidine. The 13 controls were littermates reared under conditions which were identical except that no drug was added to the diet. A distinct and significant difference in the rate of growth was first apparent 25 to 30 days after administration of the drug was started. The food intake, when calculated on the basis of body surface, ranged from 4.8 to 5.2 grams per square decimeter

during the first 12 days in both groups. During the next 45 days the control animals ate an average of 5.34 gm. per sq. dm. of body surface per day while the treated animals consumed 4.68 gm., a decrease of 31 per cent. This lessened food intake persisted, but to a lessened degree, even after the animals of both groups had ceased to gain perceptibly. Calculations of the food intake of the two groups of animals at the end of

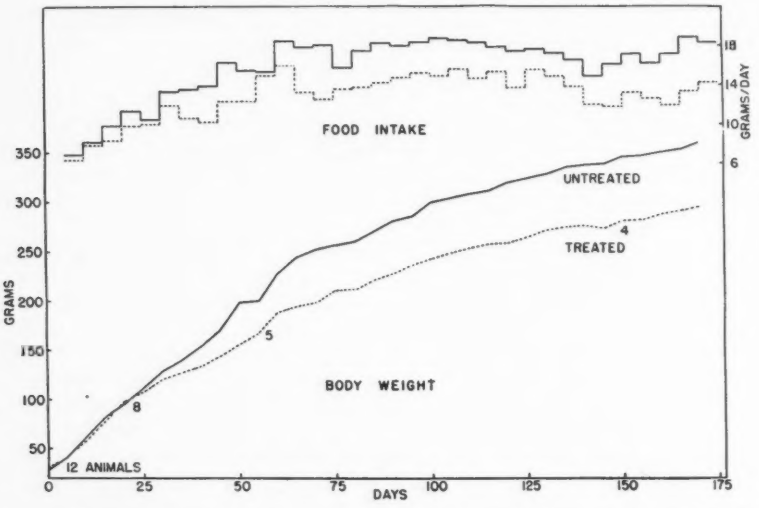

Fig. 1. Effect of adding 2 per cent by weight of sulfaguanidine to the diet on the food intake and rate of growth of normal rats, 20 days old at the beginning of the experiment. The values for food intake are expressed in gm. per rat per day, and represent averages over 5-day periods. Growth was normal in the treated group for the first 25 to 30 days, but these animals ate less food after the first 10 days, and later failed to grow normally.

the experiment showed that the treated animals consumed only 4.5 per cent less food when the comparison is made on the basis of body surface. The marked restriction of body growth, reported by Black et al. (25) in rats fed a purified diet to which sulfaguanidine had been added, has not been observed.

Hypophysectomized animals. Two groups of rats were hypophysectomized at the age of 26 days. One group of 6 animals was fed on the regular diet while the other group of 11 was given 2 per cent sulfaguanidine in their food. The body-weight curves and daily food intakes are plotted in figure 2. The drug was well tolerated and there was no perceptible difference between the weights or the food consumption in the two groups. In view of the sensitivity of hypophysectomized rats to toxins, or to stress, these findings are interpreted as indicating that the amounts of sulfaguanidine given were nontoxic. The failure of the unoperated animals given sulfaguanidine to grow normally, under the conditions of these experiments, may therefore be related to the induced state of hypothyroidism, as noted below.

Changes in the Thyroid Gland

The following description of the gross and histologic changes in the thyroid gland is based upon an examination of microscopic sections of tissue from 147 animals treated for periods of 1 to 153 days with effective quantities of the substances under

investigation. The compounds used were the above-mentioned sulfonamides[2] and thiourea, and the test animals were normal and hypophysectomized rats on the usual stock diet with and without supplements of iodine.

Gross appearance. The thyroid glands of the treated animals were enlarged and

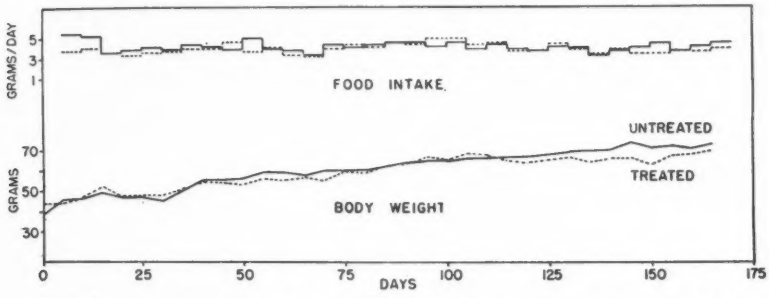

Fig. 2. Food intake and rate of growth of 8 rats hypophysectomized at 26 days of age and given 2 per cent sulfaguanidine in the diet as compared with 5 untreated hypophysectomized controls. The drug did not influence the growth or food consumption in the absence of the hypophysis.

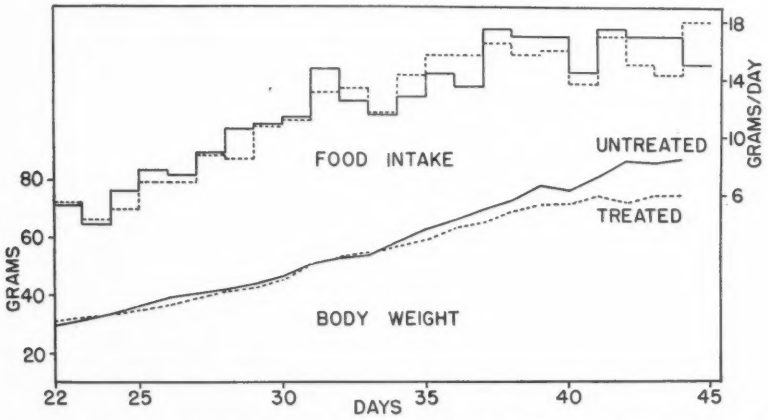

Fig. 3. Food intake and growth rate of two groups of 8 rats each given 1 per cent thyroid powder in the food. The treated animals received, in addition, 2 per cent sulfaguanidine in the diet. Neither the greatly increased food consumption, nor the decreased rate of growth was modified by the sulfaguanidine. The age of the animals is given in the abscissa.

grossly reddened (fig. 4). The enlargement involved all regions of the gland; the lobes extended laterally as well as longitudinally and an extension in a dorsal direction caused a near approximation of the two lobes behind the trachea. The isthmus was broadened and thickened and became a prominent structure which was easily visible without magnification. The redness of the gland and the enlargement of the adjoining blood vessels suggested a greatly increased blood supply.

[2] We are indebted to Dr. G. W. Clark and Dr. B. W. Carey of the Lederle Laboratores Inc. Pearl River, New York for generous supplies of sulfadiazine and sulfaguanidine, to Dr. W. A. Feirer of Sharpe and Dohme Inc. Philadelphia, Pennsylvania for the sulfasuxadine (succinyl sulfathiazole) used and to Dr. R. O. Roblin of the American Cyanamid Co., Stamford, Connecticut, for a sample of sulfanilylurea.

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When an effective level of sulfaguanidine or other active substance was administered, these changes were easily detectable within one week. The enlargement continued rapidly during the first few weeks of the experiments, but later this rapid growth decreased. The change in gross appearance at the end of 10 days of treatment is sufficiently clear cut that this period was later used in experiments to determine the relative effectiveness of the different compounds, and the minimal effective doses of each.

Histologic changes. Microscopic examination of the glands from treated animals showed a marked hyperplasia, the degree of which varied with the dose of the drug and the duration of the treatment. The first change to be noted was an increase in the height of the acinar cells followed closely by a loss of colloid from the follicles. These changes were progressive over a period of 1 to 2 weeks and were accompanied by a marked increase in the vascularity of the glands. The external diameter of the individual follicles was increased and the total number of follicles in each gland also appeared to be increased. With the disappearance of the colloid and the great increase in the size and number of the acinar cells, the follicle walls encroached upon and decreased the size of the lumina; in some places papillary projections further encroached upon the colloid-free follicular spaces (fig. 6). The central sections of the glands, normally the most active in appearance in the rat (fig. 5), were the first to show these hyperplastic changes. The large peripheral follicles were the last to be affected and they retained some colloid after the rest of the gland contained no demonstrable follicular content. An increase in the height of the follicular cells and a loss of colloid from the central portions of the gland were readily detectable 48 hours after the first dose of thiourea (fig. 8–10). A period of 1 to 2 weeks was required for the complete disappearance of colloid from the glands of animals given 0.1 per cent thiourea in the drinking water or 5 per cent sulfaguanidine in the food. After the first 2 weeks of treatment at a high dosage level no great qualitative change took place, but the glands continued to enlarge and the evidences of hyperplasia became more marked. In animals which had been treated for 2 to 6 months with a diet containing 2 per cent sulfaguanidine or with 0.1 per cent thiourea in the drinking water the glands occasionally exhibited irregularities in structure. Small areas of involution, manifested by flattening of the follicular cells and accumulation of small amounts of colloid, could be seen interspersed between markedly hyperplastic regions. A small number of follicles within the hyperplastic zones were completely devoid of follicular spaces and appeard as solid, round or oval, masses of cells which lacked normal orientation. At the end of 6 months of treatment the glands still exhibited an orderly cellular arrangement and no changes suggestive of neoplasia have been seen.

A few animals have exhibited an entirely different type of thyroid change. The

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Fig. 4. GROSS APPEARANCE OF 3 THYROID GLANDS, DORSAL ASPECT. From left to right these are: an untreated adult; a 76-day-old animal treated from the 21st day with 2 per cent sulfaguanidine in the diet; and a 65-day-old rat treated from the 21st day with 5 per cent sulfaguanidine in the diet. ×3.

Fig. 5–18. THYROID GLANDS OF FEMALE RATS. All sections were made in the same plane at right angles to the trachea and uniformly at a level near, or through, the parathyroids; hematoxylin and eosin. ×40. Fig. 5. Normal, 50-day-old rat.

Fig. 6. THIRTY-SEVEN-DAY-OLD ANIMAL WHICH HAD RECEIVED 5 per cent sulfaguanidine in the diet for 15 days. Fig. 7. FORTY-ONE-DAY-OLD RAT, HYPOPHYSECTOMIZED AT 26 DAYS of age and given 2 per cent sulfaguanidine in the food for 15 days. This gland is indistinguishable from that of an untreated hypophysectomized rat.

Fig. 8, 9, 10 and 11. GLANDS TAKEN AT 2, 4, 7 AND 12 DAYS, respectively, after the beginning of treatment with 1 per cent thiourea in the drinking water at 21 days of age. Treatment was discontinued at 7 days and figure 10 shows the degree of regression occurring in 5 days. The epithelium is flat and colloid has re-accumulated but there is little decrease in size.

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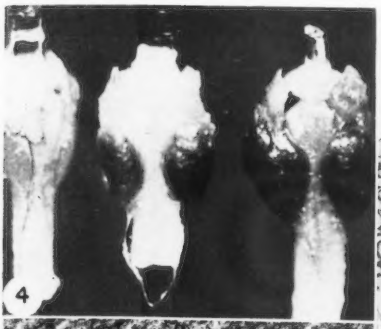
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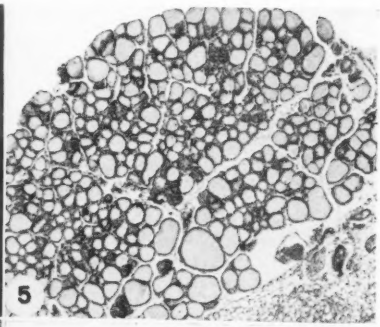
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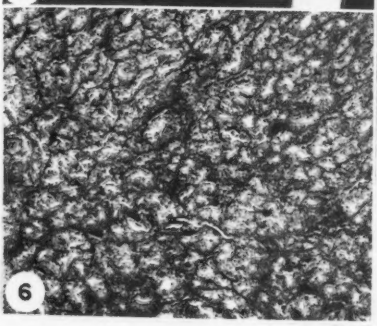
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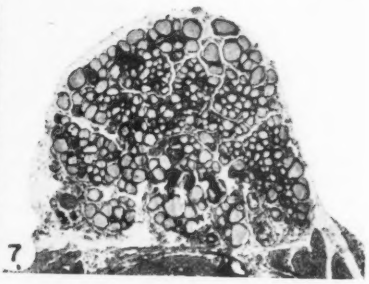
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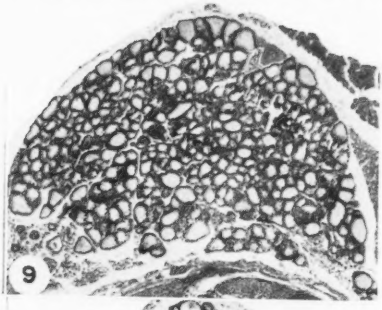
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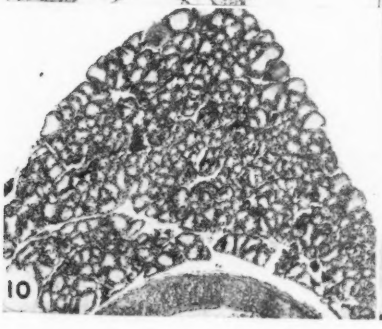
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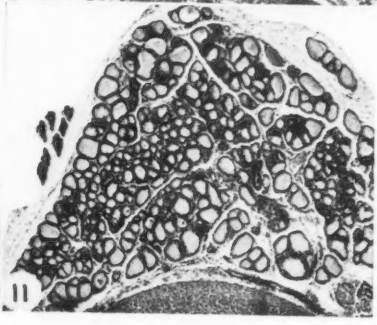
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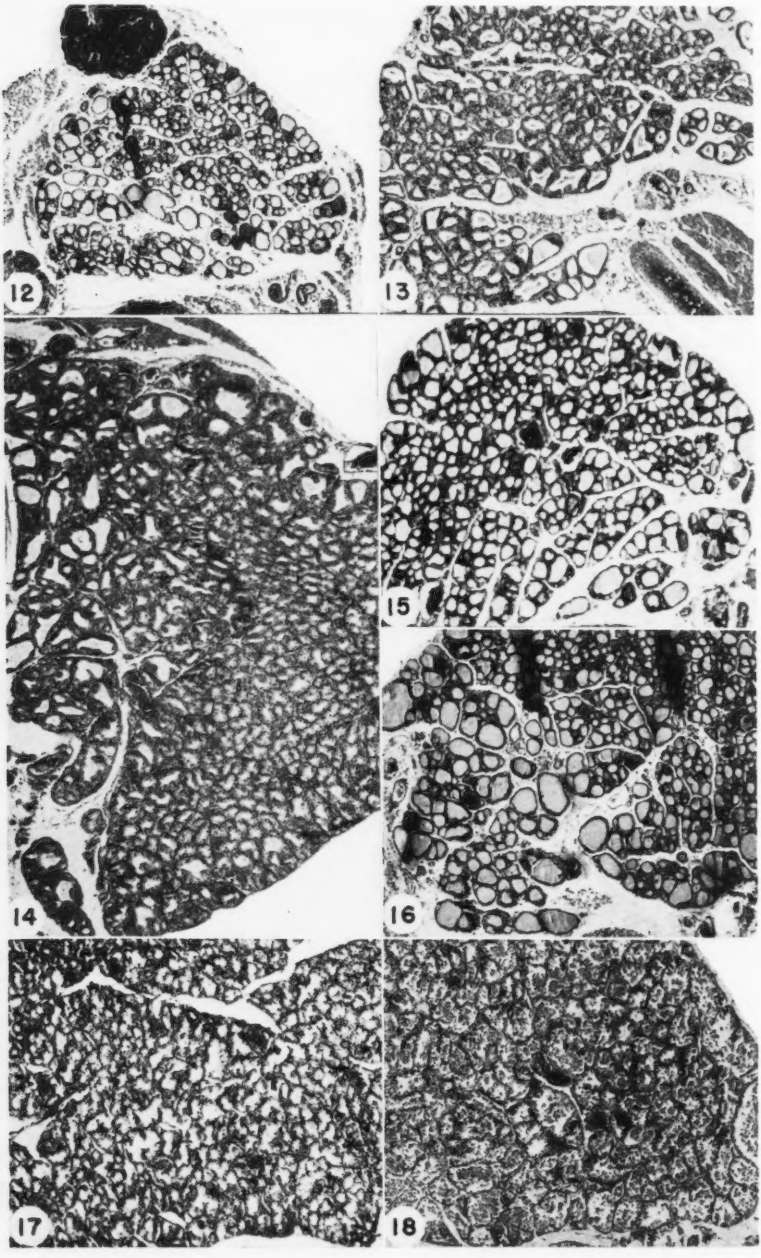
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glands were unusually large, completely free of colloid, and markedly hyperemic. They were peculiar in showing an even and diffuse process of desquamation of the follicular epithelium which involved the entire gland, leaving little to be seen of the typical picture of hyperplasia usually encountered. The follicles were uniformly large, with open lumina, and were empty except for the scattered follicular epithelial cells which appeared to be in the process of being shed from the acinar wall (fig. 18). It is not yet clear that this picture represents a qualitatively different type of response from the one usually encountered.

Upon the cessation of treatment the glands rapidly regressed toward a normal condition. Vascularity quickly decreased, the epithelium became flattened, colloid accumulated and the size of the individual follicles and the total size of the glands decreased. Precise measurements of the time required for complete return to normal structure have not been made, but these retrogressive changes appear to occur fully as rapidly as the hyperplasia. It would appear that the enlargement of the gland persists long after the histologic structure has returned to normal. Animals treated for 7 days with 1.0 per cent thiourea in the drinking water and then killed after a 5-day period without treatment exhibited gross enlargement of the thyroids but only minimal histologic evidence of the pre-existing hyperplasia (fig. 10, 11). Nine adult female rats were treated for 3 to 4 months with 2 per cent sulfaguanidine in the food and then permitted 5 days of recovery. All showed residual enlargement of the glands, but histologic evidence of regression was well marked. In 2 of the 9 animals the follicular epithelium throughout was flat and nearly all of the follicles were filled with colloid. In 2 others the glands showed a considerable irregularity in the size of the colloid-filled follicles and a few small areas showed persisting hyperplasia. The thyroids of the remaining 5 animals in this group were intermediate between these extremes in their completeness of involution.

Influence of Added Iodide

Two litter-mate groups of 5 animals each were placed on a diet containing 2 per cent sulfaguanidine at the age of 20 days. One group drank tap water, the other a 1 per cent solution of potassium iodide in tap water. Corresponding animals from the two groups were killed at intervals ranging from 7 to 30 days after the beginning of the experiment. The thyroid glands of both groups of animals exhibited a progressive hyperplasia, but the glands from the animals receiving the added iodide were not as enlarged and contained visible colloid for a longer period than did the glands of the control group. This slight degree of inhibition was also noted in two similar experiments with thiourea which was given as a 1.0 and 0.1 per cent solution, respectively,

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Fig. 12. THYROID FROM A 33-DAY-OLD RAT which had received a diet containing 2 per cent sulfaguanidine and 2 per cent thyroid powder for 13 days. The gland is atrophic and resembles the gland in figure 7. Fig. 13. EFFECT OF 10 DAYS' TREATMENT WITH 0.1 PER CENT THIOUREA and 1.0 per cent potassium iodide in the drinking water. A minimal degree of inhibition of the thiourea is seen which is considered to be an effect of toxic amounts of potassium iodide.

Fig. 14, 15 and 16. GLANDS FROM ANIMALS GIVEN 2 PER CENT SULFAGUANIDINE in the food from the 21st to 51st days of life. Fig. 14. Degree of hyperplasia induced by this treatment. Fig. 15. Gland taken 5 days after hypophysectomy, the drug being continued postoperatively. Fig. 16. Thyroid of an unoperated animal 5 days after the drug was discontinued. Figures 15 and 16 show a re-accumulation of colloid and a flattening of the follicular epithelium.

Fig. 17 and 18. GLANDS OF TREATED ANIMALS SHOWING UNUSUAL TYPES OF HYPERPLASIA. Fig. 17. An adult, treated with 1 per cent thiourea for 68 days. Fig. 18. Sixty-day-old animal which had received 0.5 per cent sulfapyridine in the drinking water for 11 days. The follicular cells appear to be breaking away from their normal attachments and floating free in the colloid-free follicular spaces.

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with and without 1 per cent of potassium iodide in the drinking water (fig. 13). It may be significant that in each case the animals receiving this large dose of iodide did not grow at all or grew less rapidly than those receiving the drugs alone. In view of the inhibitory effect of the iodide on growth, it is questionable that the iodide exerted any specific effect upon the thyroid in these experiments.

Two groups of 5 animals each were given 1 per cent thiourea in the drinking water, with and without the addition of 0.1 per cent potassium iodide. No difference in the degree of hyperplasia could be detected between these two groups of animals when they were examined after 12 and 30 days of treatment.

Consequently, iodine deficiency is a highly unlikely factor in the genesis of this form of goiter. Several thousand times the minimal daily requirement of iodine exerted no inhibitory effect upon the thyroid enlargement, and only when toxic quantities were administered did any inhibition become manifest.

Influence of Hypophysectomy

Eleven rats were hypophysectomized at the age of 26 days and given thereafter 2 per cent sulfaguanidine in the food. These animals, together with 10 hypophysectomized litter-mate controls, were killed at intervals ranging from 10 to 156 days. The thyroid glands, examined histologically, were uniformly atrophic; the follicular epithelium was flat and each follicle was well filled with colloid (fig. 7). No tendency toward hyperplasia could be seen in the glands from the animals treated with sulfaguanidine; these glands were grossly and microscopically indistinguishable from the glands of untreated hypophysectomized rats. In two other experiments using hypophysectomized rats, the animals were divided into 3 groups. An untreated group served as a control for animals receiving 0.1 or 0.5 per cent thyroid powder in the food, with or without 2 per cent sulfaguanidine. In all cases the thyroid glands were atrophic and showed no effects either of the sulfaguanidine or of the thyroid feeding.

Seventeen rats were hypophysectomized at 26 days of age and divided into two groups. One group received the usual diet and the other this diet to which 2 per cent sulfaguanidine had been added. After 5 days, injections of 25 mg. of whole pituitary powder from sheep were made daily for 10 days into 6 animals of each of the above groups, while the remaining animals were uninjected. This dose of pituitary powder induced a slight enlargement of the thyroid, an increase in the follicular-cell height and a slight loss of colloid. The thyroid glands of the animals receiving sulfaguanidine could not be distinguished histologically from those of the injected animals not receiving the drug. It was clear that the intact hypophysis was essential to the goiterigenic action of these compounds.

Three animals which had received sulfaguanidine from the 21st to the 47th day of age at a level of 2 per cent in the food were hypophysectomized and continued on treatment with the drug for a period of 5 days. Two unoperated animals, treated in the same way, exhibited markedly hyperplastic glands while the hypophysectomized animals showed a pronounced degree of thyroid regression (fig. 14, 15). The follicular cells were flat and all follicles were well filled with colloid; the glands had decreased in size, but were still somewhat larger than the normal. The regression noted in these animals after hypophysectomy was more nearly complete than that observed in unoperated rats following the withdrawal of the drug (fig. 16).

Effect of Thyroid Feeding

Desiccated thyroid powder was admixed with the food in amounts of from 0.1 to 2.0 per cent by weight. Experiments were performed on normal and hypophysectomized rats to determine *a*) the influence of thyroid feeding on the goiterigenic action

of the drugs; and *b*), the influence of these drugs on the calorigenic effect of the thyroid powder.

Two groups of 6 litter-mate rats each were given 2 per cent thyroid powder in their food from the age of 20 days. One of the two groups was given 2 per cent sulfaguanidine in addition to the thyroid powder. Seven of the 12 animals died between the 9th and the 13th day of this regimen; the 5 remaining animals were killed on the 14th day. There was no significant difference between the two groups in the rate of growth, the daily food intake or the length of survival. The thyroid glands of both groups were uniformly atrophic, resembling the glands of hypophysectomized rats (fig. 12).

This experiment was repeated using two groups of 8 litter-mate rats each given 1 per cent thyroid powder in the food from the age of 22 days. As before, one group also received 2 per cent sulfaguanidine in the food. Deaths occurred between the 9th and

TABLE 1. EFFECT OF SULFAGUANIDINE ON THE CALORIGENIC ACTION OF ADMINISTERED THYROID POWDER IN NORMAL IMMATURE RATS

Treatment	Number of Animals	Metabolic Rate, cal./sq.m./hr.			
		No. of determ.	Range	Mean	S.E.
1% Thyroid powder 2% Sulfaguanidine	5	15	52.1–86.0	74.9	±2.8
1% Thyroid powder	5	21	54.7–87.6	71.6	±2.4

the 25th day in both groups, and on the 25th day the 2 remaining animals in the group receiving thyroid alone were killed. Again, the sulfaguanidine was noted to offer no protection against the lethal effect of thyroid powder or against its calorigenic action as measured by the food intake. The growth rate of these animals is shown in figure 3 which shows that the sulfaguanidine did not inhibit the toxic action of the thyroid powder as reflected in the inhibition of growth. Measurements of the metabolic rates of 10 of these animals made between the 14th and the 22nd days of treatment showed that no significant difference existed in the rate of oxygen consumption in the two groups (table 1). In both groups the thyroid powder greatly increased the metabolic rate. Sections of the thyroid glands in these animals showed them to be atrophic, and no trace of the goiterigenic effect of the sulfaguanidine could be detected in the drug-treated animals.

Two groups of 11 animals each were hypophysectomized at the age of two months when they weighed 103 to 141 grams. Twelve days later both groups were placed on a diet containing 0.1 per cent thyroid powder, one group with, and one without, an additional 2 per cent sulfaguanidine. After 7 days, the average body weight of the animals receiving the sulfaguanidine had fallen from 110.9 to 93.5 gm. while the other group lost from 109.0 to 95.7 gm. During the next 17 days 5 of the former and 4 of the latter group had died and weight loss had continued, but during this period there was too great a variation in body weight within the two groups to permit an accurate analysis. The animals treated with sulfaguanidine, on the whole, lost more weight and ate less food than the control group, and there was no indication that the sulfaguanidine afforded protection against the toxic or calorigenic actions of the thyroid powder.

These experiments indicated that sulfaguanidine was ineffective in inhibiting the calorigenic action of artificially administered thyroid hormone. It was apparent, how-

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ever, that thyroid hormone completely prevented the goiterigenic effect of sulfaguanidine.

Changes in the Metabolic Rate

Measurements on the rate of oxygen consumption were made on a number of animals during the course of the experiments recorded above. Care was taken to obtain readings when the animals were resting quietly, and some of the measurements were made during periods when the animals were actually asleep. Despite attempts to maintain constant conditions, there were wide variations from day to day in the measurements on individual animals. The lack of constancy in these results detracted from the confidence that could be placed in the absolute values obtained. However, when measurements were made at the same time on test animals, and on appropriate controls, the comparative values were found to be sufficiently consistent to warrant a discussion here of the results thus far obtained.

Determinations on the animals whose rate of growth is shown in figure 1, and which received 2 per cent sulfaguanidine in the food, were initiated on the 47th day

TABLE 2. METABOLIC RATE OF A RAT TREATED WITH 0.1 PER CENT THIOUREA IN THE DRINKING WATER COMPARED WITH THAT OF A LITTER-MATE UNTREATED CONTROL

Days treated	0	7	11	17	21	27
B.M.R., %	−4.3	−3.4	−9.3	−3.6	−11.5	−30.5

TABLE 3. METABOLIC RATE IN CALORIES/SQ.M./HR. OF A RAT TREATED WITH 0.1% THIOUREA IN THE DRINKING WATER AND OF AN UNTREATED LITTER-MATE CONTROL. ON THE 13TH DAY OF THE EXPERIMENT BOTH ANIMALS WERE PLACED IN A COLD ROOM AT 4°C.

Days treated	0	5	8	11	15	18	22	26
Days after exposure to cold					2	5	9	13
Control	39.2	40.6	36.8	41.0	48.8	49.0	46.9	42.6
Treated	39.4	40.8	36.4	41.0	47.0	41.4	39.2	36.0
Difference, %	+0.5	+0.5	−1.1	±0	−1.7	−15.5	−16.4	−15.5

of treatment. At this time the basal metabolic rate of the treated animals had fallen to levels which were consistently lower than those of the controls. This observation was confirmatory to the index to lowered metabolism given by the measurements of the food intake. A number of experiments were therefore made to determine the time required for the development of this decrease in metabolism.

Eleven animals were injected subcutaneously with a suspension of sulfaguanidine in sesame oil; each animal received 0.1 gm. every two days, the metabolic rates being measured 24 hours after each injection and compared with that of 5 uninjected controls. During the 9 days following the first injection no significant changes in the rate of oxygen consumption were observed. A second similar experiment was carried out over a period of 8 days. Two animals received 1 per cent thiourea in the drinking water during this period while 2 litter-mate controls were untreated. No significant alteration in the metabolic rate, measured every 2 days, resulted in this period of time.

Subsequent experiments have confirmed that little or no change in the rate of basal oxygen consumption is to be observed over the first 7 to 14 days of treatment with these substances. With more prolonged treatment, however, with effective doses of active compounds, a distinct decrease in basal metabolic rate takes place. Two illustrative experiments on animals given thiourea as a 0.1 per cent solution in the drinking water are recorded in tables 2 and 3. In table 2 the metabolic rate of a treated animal, expressed as a percentage of that of its untreated litter-mate, is shown to remain nor-

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mal for the first 17 days. By the 27th day the basal metabolism of this animal had fallen to −30.5 per cent. In table 3 are shown the rates of metabolism of 2 similar litter-mate animals which were placed in a cold environment on the 13th day of the experiment. Both animals exhibited elevated metabolic rates under these conditions, even though measurements were made several hours after they were returned to the warm room. The treated animal exhibited a consistently lowered rate of metabolism

TABLE 4. RELATIVE ACTIVITIES OF THE SULFONAMIDES AND THIOUREA

Drug	Method of Administration	Duration in Days	Blood Level, mg. %		Degree of Thyroid Hyperplasia
			Range	Mean	
Sulfadiazine	0.01%, water	12			None
	0.02%, water	11	0.9–2.8	1.7	Minimal
	0.10%, water	11–18			Maximal
	1.00%, food	12	32.2–42.2	32.4	Maximal
Sulfapyridine	0.1%, water	18			Minimal
	0.5%, water	11	1.0–6.0	4.1	Maximal
	1.0%, food	19			Maximal
Sulfathiazole	0.1%, water	18			None
	0.5%, water	11	1.6–4.5	3.3	Minimal
	1.0%, food	19			Moderate
Sulfanilylurea	0.2%, food	14			Moderate
Sulfaguanidine	50 mg., sub. cut.	25–60	2.9–4.9	3.7	Minimal
	125 mg.	25–60	4.6–7.7	6.3	Moderate
	500 mg.	10–25	20.6–23.6	22.7	Maximal
	0.1%, water	10			None
	0.5%, food	11			Moderate
	2.0%, food	7–153	6.7–12.9	10.0	Moderate
	5.0%, food	7–44	15.4–26.4	22.2	Maximal
Sulfanilamide	0.1%, water	18			None
	0.5%, water	11	6.7–15.1	10.0	Minimal
	2.0%, food	7–35			Moderate
Sulfasuxadine	5.0%, food	10			Minimal
Thiourea	0.01%, water	18			Minimal
	0.02%, water	11			Minimal
	0.10%, water	10–18			Maximal
	1.00%, water	7–68			Maximal
	2.00%, food	7–35			Maximal

from the 5th day of cold exposure onward and died 3 days after the end of the experiment. Other experiments, as yet of a preliminary nature, indicate that in a cold environment a more marked and much more consistent decrease in the metabolic rate follows the administration of these compounds. A limited series of experiments on thyroidectomized animals indicates that the degree of lowering of the basal metabolic rate, subsequent to complete removal of the thyroid gland, is quite small when the animals are housed in warm quarters. It is quite possible, therefore, that the degree of thyroid dysfunction induced by these compounds is considerably greater than is indicated by the above figures for the basal metabolic rate.

Relative Activities of the Sulfonamides and Thiourea

A measure of the relative potencies of these compounds in inducing thyroid hyperplasia was obtained by administering the different substances in the food or water

at several dosage levels. When the test animals were killed after a period of 7 to 18 days the degrees of thyroid enlargement and hyperemia were graded as negative, minimal, moderate or maximal. A second gradation of effect was obtained from histologic sections of the glands. Agreement between these two criteria was good, and as the rating of each gland was made in most cases without knowledge of the antecedent treatment, the method was adequately objective.

In table 4 it may be seen that sulfadiazine proved to be by far the most active sulfonamide tested. As low a concentration as 0.02 per cent in the drinking water was adequate to induce a detectable effect in 11 days. The average daily intake of drug in these animals was 4.2 mg. per 100 grams of body weight, and the resulting levels of free sulfonamide in the blood averaged 1.4 mg. per cent, a level that is not distinguishable, with certainty, from zero with the method used. Sulfapyridine induced a minimal effect when given as a 0.1 per cent solution; 18.9 mg. per 100 gm. of body weight was taken daily, and an average blood level of 4.1 mg. per cent was obtained. The average daily oral doses in mg. per 100 gm. of body weight for a minimal thyroid effect with the other sulfonamides were: sulfathiazole, between 21.7 and 69.0; sulfanilylurea, approximately 40; sulfaguanidine, 51.0; sulfanilamide, 70.4; sulfasuxidine, about 500.

The relatively low potency of sulfaguanidine is partially attributable to its poorer absorption from the intestinal tract. However, even when injected subscutaneously as a suspension in oil, a daily dose of 50 mg. produced only a minimal response. The very low activity of sulfasuxidine may be due either to its poor absorption or to its chemical configuration. It is of interest that sulfadiazine is effective at blood levels far below those that are effective against bacterial infections. Sulfathiazole and sulfapyridine are also active at blood levels below those usually maintained in patients, while sulfanilamide requires somewhat higher blood levels for a full thyroid effect.

Thiourea exerted a minimal effect when 3.3 mg. per 100 gm. of body weight per day were given. Thus, on a weight basis, it is slightly more active than sulfadiazine, but on a molar basis sulfadiazine is the more active compound.

DISCUSSION

It is clear from the foregoing results that certain sulfonamide compounds and thiourea, when administered to normal rats in doses that are not in the usual sense to be considered toxic, induce a pronounced enlargement of the thyroid gland. The histologic changes are consistent with those generally recognized as typical of hyperplastic goiter and do not differ, except perhaps in degree and in rate of development, from those resulting from acute iodine deficiency or from those seen in human toxic goiter. In spite of the histologic picture of marked overactivity, there are no signs of hyperthyroidism; in fact, after a variable period of time, the animals whose thyroid glands are markedly hyperplastic begin to exhibit distinct signs of hypothyroidism i.e. subnormal growth and development, lessened food intake and lowered basal oxygen consumption.

The co-existence of thyroid hyperplasia and lowered rate of metabolism suggested that the mechanism of action of these compounds should be sought within two possible realms. The drug either exerted an action which inhibited the normal effect of the thyroid hormone on tissue in general, or else it interfered in some way with the adequate production of normal thyroid hormone. In either case the goiter would be the result of a compensatory hyperplasia which could not completely make up for the induced deficit.

The fact that no evidence could be educed to indicate any degree of hyperthyroidism at any time during the course of the administration of these drugs was taken as

strong evidence against the possibility that the compounds either exert a direct hyperplastic stimulus to the thyroid gland or exert a stimulating action on the anterior pituitary resulting in an increased output therefrom of thyrotropic hormone. These two possibilities were equally unlikely unless the drug were to have at least two quite different actions, the one inducing thyroid hyperplasia directly or over the anterior pituitary, and the other directed toward the lowering of the metabolic rate by one means or another.

A more complete understanding of the processes controlling normal thyroid function would aid in the interpretation of the mechanism of the goiterigenic effect of these compounds. In full recognition of its inadequacies, the following concept of normal thyroid physiology forms the basis for the present discussion. The basic system governing the structure and endocrine function of the thyroid appears to be an interrelation and balance between the thyrotropic activity of the anterior pituitary and the production of a calorigenic substance from the thyroid gland. Thyroid hormone is thought to decrease the output of thyrotropin while a lack of thyroid hormone, or the consequent lowering of metabolism, is considered to be a normal stimulus to the release of thyrotropin from the hypophysis. In this way a balance is reached which maintains the metabolic rate of the organism within a normal range. Within this restricted range, normal thyroid activity is thought to fluctuate under the influence of other factors which may raise or lower thyroid function either directly or indirectly through the pituitary. These other factors, including environmental and seasonal changes, alterations in physiological functions, and nervous and mental influences are not pertinent to the present discussion.

In an interpretation of the data which have been presented, a clearer understanding results when it is assumed that a single hypophyseal factor is concerned with both hyperplasia and oversecretion in the thyroid. The data indicate that the histologic picture of hyperplasia consistently parallels a loss of colloid, and that these two phenomena are considered to be the combined result of thyrotropic stimulation. The accumulation of colloid in the atrophic gland of the hypophysectomized animal is taken to mean that colloid storage is not brought about through any pituitary stimulus but is, in fact, an index of a lack of thyrotropic stimulation. When the pituitary is removed from an animal in which the thyroid is hyperplastic and colloid-free, colloid rapidly accumulates in the regressing gland (cf. fig. 14, 15).

The former of the two probable mechanisms of action of these substances, i.e. an inhibition of the action of the thyroid hormone upon tissue in general, was the subject of several of the experiments reported above. Artificially administered thyroid hormone proved to be equally calorigenic and toxic in the drug-treated animals as in the controls. This was seen both in normal and in hypophysectomized rats, and was taken as evidence against an antagonistic action of the drug upon the thyroid hormone and its activity.

That the action of these drugs does not involve a direct hyperplastic stimulus to the thyroid gland was indicated by the experiments on hypophysectomized rats. In the absence of the pituitary the thyroid glands became atrophic and the goiterigenic action of these compounds was completely lacking. In answer to the possibility that the thyroid could not respond to a postulated stimulus from these goiterigenic agents in the absence of all pituitary hormone action, whole pituitary powder was administered to hypophysectomized rats. Under these conditions the thyroid glands remained in a normal condition, or were slightly overstimulated, but here again neither sulfaguanidine nor thiourea produced any visible change in the gland.

The results of the feeding of thyroid powder are strongly in favor of the view that the thyroid hyperplasia is brought about by an overproduction of thyrotropin. The

administration of thyroid hormone to normal animals is known to depress the thyrotropic activity of pituitary. In these experiments the thyroid glands of normal animals were rendered atrophic by this treatment, and the goiterigenic effect of both sulfaguanidine and thiourea was completely inhibited. In the normal animal, therefore, the increased production of thyrotropin in response to these drugs is considered to be a consequence of a subnormal amount of thyroid hormone reaching the pituitary—when this deficit is artificially made up by thyroid feeding the goiterigenic action of the drug is abolished. Were the drug itself a stimulus to the pituitary to release thyrotropin, one would expect a state of hyperthyroidism to ensue and one would not anticipate that thyroid hormone would effectively inhibit the resulting thyroid hyperplasia.

In view of these considerations it may be concluded that the mechanism of the goiterigenic action of the substances investigated resides in an intereference in the synthesis of thyroid hormone. The experiments reported above do not permit of speculation on the details of this inhibition, but it can be assumed that one or more of the many steps in the manufacture of the normal thyroid hormone is in some way hindered. The action is most probably centered within the thyroid gland itself, but there is no evidence against the alternative that the action is elsewhere and concerned with the synthesis of some essential component or precursor of the hormone. In either case the effect may be upon an enzyme system, but, whatever the eventual site of inhibition, the action must be a highly specific one because, as far as the above data go, there is little to indicate that any other physiologic function is seriously modified.

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SUMMARY

The administration of certain sulfonamides, or of thiourea, to normal rats resulted in an enlarged, hyperemic and hyperplastic thyroid gland, coincident with a state of hypothyroidism as indicated by decreased food intake, decreased growth and development and by lowered basal oxygen consumption. The thyroid hyperplasia was not influenced by large supplements of iodine, but was abolished by the administration of thyroid powder and by hypophysectomy. The calorigenic action of thyroid powder in normal or in hypophysectomized rats was not inhibited by these compounds. The goiterigenic activity of the compounds tested decreased in the order: thiourea, sulfadiazine, sulfapyridine, sulfathiazole, sulfaguinidine, sulfanilylurea, sulfanila-

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mide and sulfasuxidine. The thyroid hyperplasia which occurs under the influence of these drugs is considered to be compensatory to the failure of thyroid hormone synthesis.

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CORRECTED EVALUATION OF THE RESULTS OBTAINED WITH SHAW'S COLORIMETRIC 'ADRENALIN' METHOD

THE WRITER's original belief that hormonal steroids form compounds with adrenalin and thus participate directly in the results obtained with Shaw's method[1–4] has been abandoned since it was found that it is not possible to hold steroids (desoxycorticosterone acetate and a cortical extract prepared by Dr. E. C. Kendall) in aqueous solution together with even large amounts of adrenalin. The steroids passed quantitatively into organic solvents, such as chloroform and petroleum ether, while the adrenalin remained in the water. Nevertheless, the entire chromogenic material found with the routine procedure in adrenal glands and in blood was quantitatively recovered in chloroform extracts of dried adrenal glands and blood, with the specific ratio unchanged. This seems to be due to the fact that adrenalin becomes chloroform-soluble in the presence of lecithin[5] which was confirmed by the writer and also found to hold true for adrenalone and ascorbic acid.

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Colorimetric results with a specific ratio analogous to that characteristic for pure adrenalin (or sympathin[6]) were frequently obtained in human arterial walls and in striated muscles of the rat, less frequently in human hearts and kidneys.

In most tissues, including the adrenal glands and blood, however, a lower denominator of the specific ratio indicated the participation of substances other than adrenalin (or sympathin) in the colorimetric results. Since the denominator of the specific ratio of ascorbic acid is 0.3, this substance can be assumed to contribute to the relatively low denominators of the specific ratio found in most tissues and in blood. However, the amounts of ascorbic acid which are normally present in these tissues, according to the literature, do not suffice to account for the values actually found, the color intensity of ascorbic acid being quite weak (1/320 of that of adrenalin).

In the search for other interfering chromogenic substances with a lower denominator of the specific ratio than that of adrenalin it was found that the principal requirement, namely adsorbability by $Al(OH)_3$ at a pH of 8.5 which is essential for participation in the final color reaction was fulfilled only by those of the many substances tested which contained at least two adjacent hydroxyl groups on a benzol ring.

The following substances related to adrenalin part of which are believed to exist in adrenal tissue, were recovered by the second adsorption in Shaw's method: adrenalin, dihydroxyphenylalanine, adrenalone, leukoadrenochrome (?), epinine, pyrocatechol, ascorbic acid; furthermore a substance probably identical with sympathin.[6] Other adrenalin derivatives

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deprived of the catechol nucleus (quinones, adrenochrome) and a variety of reducing substances not related to adrenal function were not adsorbed by $Al(OH)_3$ at a pH of 8.5 and, consequently, did not participate in the final reaction.

The conclusion is reached that Shaw's method is not specific for adrenalin but that it includes beside ascorbic acid a number of adrenalin precursors and derivatives with a catechol nucleus. Whether or not any other biologic substances participate is uncertain. None has been found so far. The specific ratio permits a rough estimate regarding the prevalence or non-prevalence of adrenalin proper within the total readings. This was demonstrated by an increase of the denominator of the specific ratio of various tissues and of blood after injection of adrenalin and, according to Cannon and Lissak[6] by its decrease after sympathetic denervation.

In view of the non-specificity of the method for adrenalin and considering the inclusion of other related catechol compounds in the results, attempts were made to investigate the possible specificity of the method as a criterion of adrenal hormone production and secretion in a broader sense than concerning the adrenalin fraction alone. The adrenalin-like sympathomimetic action of certain catechol compounds with a low denominator of the specific ratio has been established by other investigators[7,8,9] and the catechol nucleus which was found to be decisive for appearance in the colorimetric results is also believed to determine both the sympathomimetic action of adrenalin and related compounds[7] and their high electric potential.[10]

Extensive studies were made with the method in tissues and blood of animals and man in order to correlate the chemical results with existing knowledge and conceptions of adrenal physiology and pathology.

The content of adsorbable chromogenic material of the adrenal glands was found to be very much higher than that of any other tissue, the concentration in the medulla was 8 to 10 times greater than in the cortex. Increases of the total readings and a behavior of the denominator of the specific ratio according to expectation were observed in various tissues and in blood after injection of adrenalin, adrenalone and dihydroxyphenylalanine and under experimental conditions which are known or believed to be accompanied by increased adrenal activity, such as physical exercise, exposure to cold, administration of insulin, and in clinical conditions such as adrenal tumors, pulmonary edema, angina pectoris. Diminished readings, on the other hand, were obtained after adrenalectomy, after roentgen-ray irradiation of the adrenal glands and in the heart of one human case with denervated adrenal glands. Some analogous observations have also been reported by Cannon and Lissak[6] and by Bullen and Bloor.[11]

The order of concentration of chromogenic material other than adrenalin in the organs of the rat corresponds closely to the order of concentration of the amine-oxidase which causes deamination of the side-chain of adrenalin leaving the catechol nucleus intact.[12] It also corresponds to the order of efficiency of these organs in removing adrenalin from perfusing fluids[13,14] in the following succession: liver, kidney, brain, muscle.

Summary.—The writer's original belief that steroids participate directly in the results obtained with Shaw's colorimetric 'adrenalin' method has been abandoned. No compound formation with adrenalin seems to take place although the chromogenic and biologic properties of adrenalin are altered by certain steroids.

A number of substances related to adrenalin and containing 2 hydroxyl groups in ortho-position were found to participate in the reaction. Whether or not other substances beside ascorbic acid, unrelated to adrenal and adrenergic function and adsorbable by $Al(OH)_3$ at a pH of 8.5, participate in the readings obtained from tissues and blood cannot be stated at the present time.

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In the adrenal glands themselves and most other tissues the presence of chromogenic and adsorbable substances other than adrenalin was indicated by the specific ratio. In certain tissues, however, the specific ratio indicate the presence of uncontaminated adrenalin (or sympathin).

The behavior of the colorimetric results obtained in tissues and blood under various experimental and clinical conditions was found to agree in general with existing knowledge and conceptions regarding adrenal physiology and pathology.

W. RAAB

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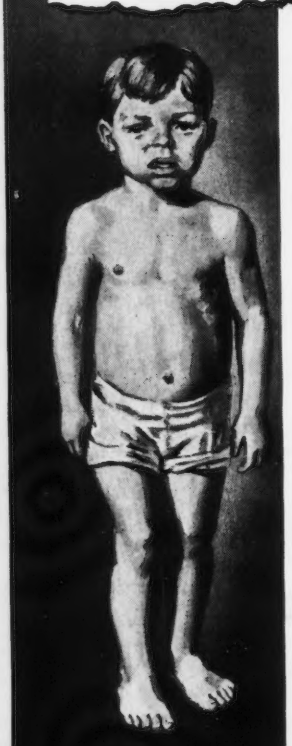

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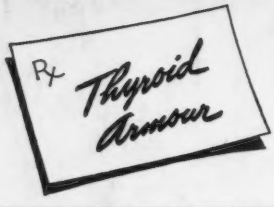

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In view of the non-specificity of the method for adrenalin and considering the inclusion of other related catechol compounds in the results, attempts were made to investigate the possible specificity of the method as a criterion of adrenal hormone production and secretion in a broader sense than concerning the adrenalin fraction alone. The adrenalin-like sympathomimetic action of certain catechol compounds with a low denominator of the specific ratio has been established by other investigators[7,8,9] and the catechol nucleus which was found to be decisive for appearance in the colorimetric results is also believed to determine both the sympathomimetic action of adrenalin and related compounds[7] and their high electric potential.[10]

Extensive studies were made with the method in tissues and blood of animals and man in order to correlate the chemical results with existing knowledge and conceptions of adrenal physiology and pathology.

The content of adsorbable chromogenic material of the adrenal glands was found to be very much higher than that of any other tissue, the concentration in the medulla was 8 to 10 times greater than in the cortex. Increases of the total readings and a behavior of the denominator of the specific ratio according to expectation were observed in various tissues and in blood after injection of adrenalin, adrenalone and dihydroxyphenylalanine and under experimental conditions which are known or believed to be accompanied by increased adrenal activity, such as physical exercise, exposure to cold, administration of insulin, and in clinical conditions such as adrenal tumors, pulmonary edema, angina pectoris. Diminished readings, on the other hand, were obtained after adrenalectomy, after roentgen-ray irradiation of the adrenal glands and in the heart of one human case with denervated adrenal glands. Some analogous observations have also been reported by Cannon and Lissak[6] and by Bullen and Bloor.[11]

The order of concentration of chromogenic material other than adrenalin in the organs of the rat corresponds closely to the order of concentration of the amine-oxidase which causes deamination of the side-chain of adrenalin leaving the catechol nucleus intact.[12] It also corresponds to the order of efficiency of these organs in removing adrenalin from perfusing fluids[13,14] in the following succession: liver, kidney, brain, muscle.

Summary.—The writer's original belief that steroids participate directly in the results obtained with Shaw's colorimetric 'adrenalin' method has been abandoned. No compound formation with adrenalin seems to take place although the chromogenic and biologic properties of adrenalin are altered by certain steroids.

A number of substances related to adrenalin and containing 2 hydroxyl groups in ortho-position were found to participate in the reaction. Whether or not other substances beside ascorbic acid, unrelated to adrenal and adrenergic function and adsorbable by $Al(OH)_3$ at a pH of 8.5, participate in the readings obtained from tissues and blood cannot be stated at the present time.

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[12] Blaschko, H., D. Richter and H. Schlossmann: *Biochem. J.* 31: 2187. 1937.
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In the adrenal glands themselves and most other tissues the presence of chromogenic and adsorbable substances other than adrenalin was indicated by the specific ratio. In certain tissues, however, the specific ratio indicate the presence of uncontaminated adrenalin (or sympathin).

The behavior of the colorimetric results obtained in tissues and blood under various experimental and clinical conditions was found to agree in general with existing knowledge and conceptions regarding adrenal physiology and pathology.

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